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A COMMON DENOMINATOR IN AGING RESEARCH

JOHAN BJORKSTEN*

We live in an era of research successes. The following charts will illustrate that point. (37) (Fig. 1) The speed attainable by man has increased from the speed of the horse-and-buggy days up to space speeds. The maximum amount of power attainable by man has increased from muscle power to Watt steam engine, and has now reached the nuclear range. The amount of electric power available to man has gone up in a similar ratio following the increase in overall research efforts from something nominal only a few decades ago up to eight billion a year or more today.

Against this backdrop let us look at the following chart, which illustrates the longevity of man and woman at sixty from 1789

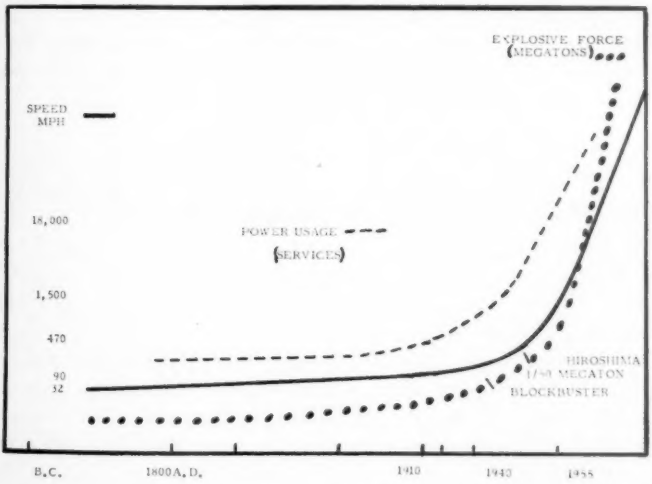

FIG. 1. Curves showing exponential rate of progress in fields subjected to determined research efforts.

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till 1957. (32) (Fig. 2) We see here that while in all of the other fields subjected to intense research there has been exponential progress, the improvement in human longevity above 60 years has been trifling in comparison. The increases in lifespan so far are due to improvements in infant mortality and to control of infectious disease at middle age—but the chances of a man of sixty to live on are only two years better on the average now than they were 150 years ago. Why this lag?

In part, it is because of the tendency to take aging for granted as something natural, unavoidable, and therefore, very little is done. Then, another reason may be the tendency to spend funds on small, uncoordinated efforts rather than on a major, frontal attack with determination to push through to a success. The third reason is that so much effort has been spent on the specific aspects of aging in this organ, that organ; this phenomenon, that phenomenon; which is all very good and very valuable and very necessary. But what is needed now perhaps is to take all this data that has been accumulated and see if we cannot find a common denominator; in erecting a building we need not only the building blocks but also a common mortar to tie them together.

Let us first view the mortality curve and analyze it, because the mortality curve is a good, general expression of aging in its relation to longevity. (Fig. 3) In this curve, age, or time, is plotted

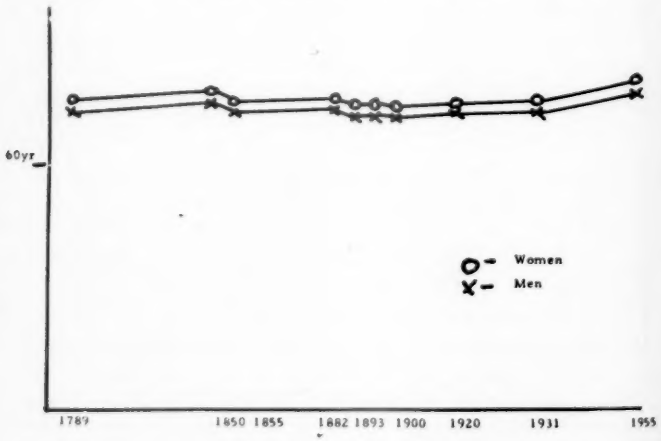

FIG. 2. Life Expectancy at Age 60.

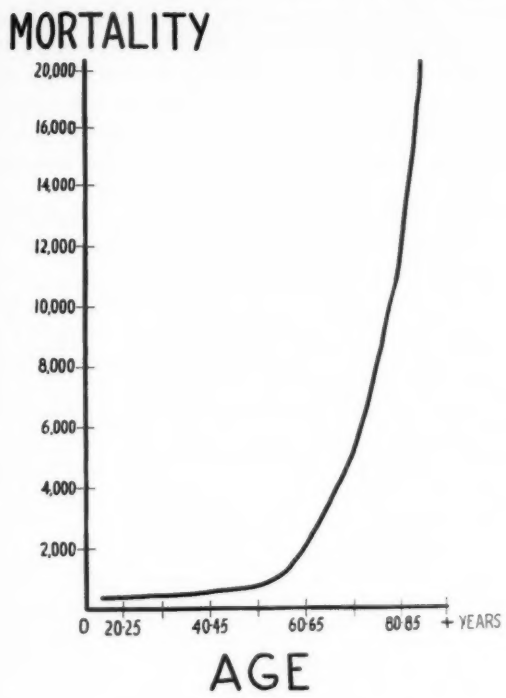

FIG. 3.

against mortality. Out of 100,000 boys of 15 years, about 100 will die in one year. Out of 100,000 men of 85, about 20,000 will die in one year, and the increase is exponential. Certainly the traumata encountered by these people are about the same from one year to another, plus or minus a couple hundred percent, maybe, but it is certainly not an exponential increase like this. So, what this curve really means is an expression of the decrease in resistance to all types of traumata. Now, if there is a common cause, if there is indeed a common cause of the changes that occur in aging, then this common cause should obey the same laws as the mortality curve and should be expressable with the same sort of terms and have a curve of the same general shape.

Because of facts which I shall detail later, there is good reason to believe that the one common denominator in aging might be a

progressive insolubilization of large molecules. Therefore, we took some protein material—this happens to be a gelatin—and immobilized it by means of a cross-linking agent, and drew up a curve of the progressive phenomena. This curve is shown below. (Fig. 4) The similarity is striking if you compare this with the mortality curve. Furthermore, if both curves are plotted on semi-

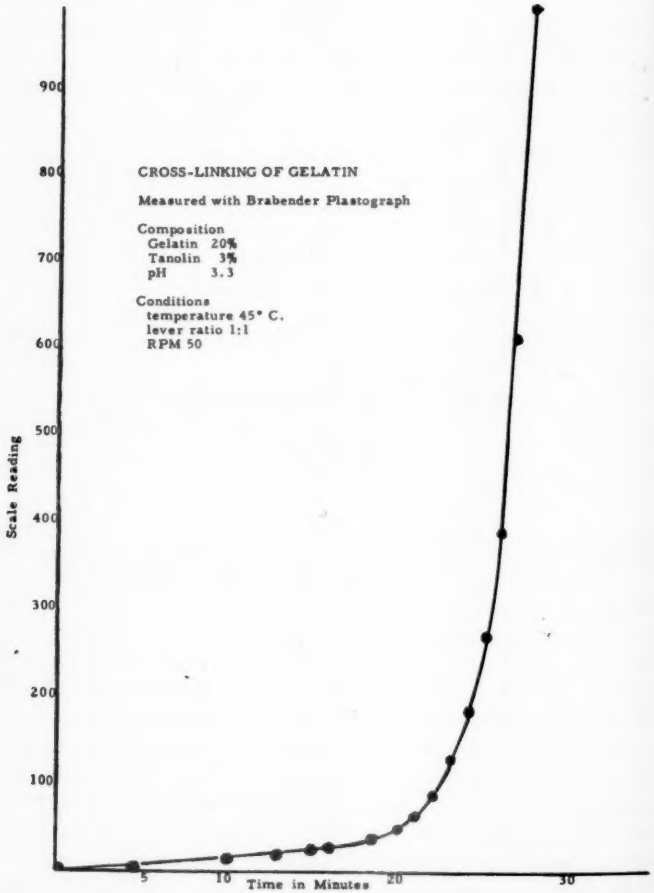

FIG. 4.

log paper, both will show a similar sharp break. In the mortality curve, we note progress at one rate up to about age 35 and then it bends and progresses at an accelerated rate. This peculiar break also occurs in the curve of viscosity against time in protein being cross-linked by a cross-linking agent. (Figs. 5 and 6) (12)

Aside from the striking resemblance of these curves, what indications do we have that there is any relationship? First there is the proven fact that a large number of potential cross-linking agents are normal metabolites or are otherwise normally present in the human bloodstream. (1, 16, 20–27, 31, 35, 36, 38, 39, 46, 47, 50)

Second, it has been shown that a protein which has been so cross-linked is impaired in its hydrolysis so that the enzymes will not hydrolyze it again. (5) (28) Therefore, an immobilization by such means is permanent. The protein which has been im-

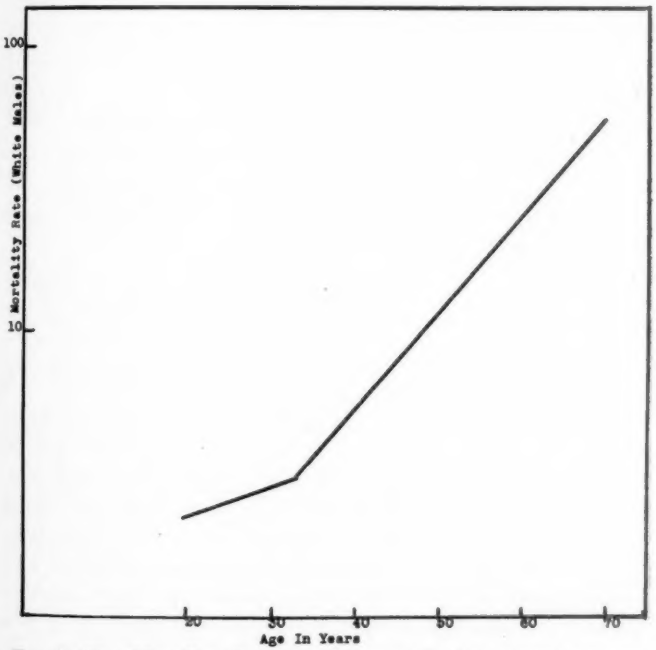

FIG. 5. The Mortality Curve for Humans, FIG. 3, plotted on semi-logarithmic graph paper.

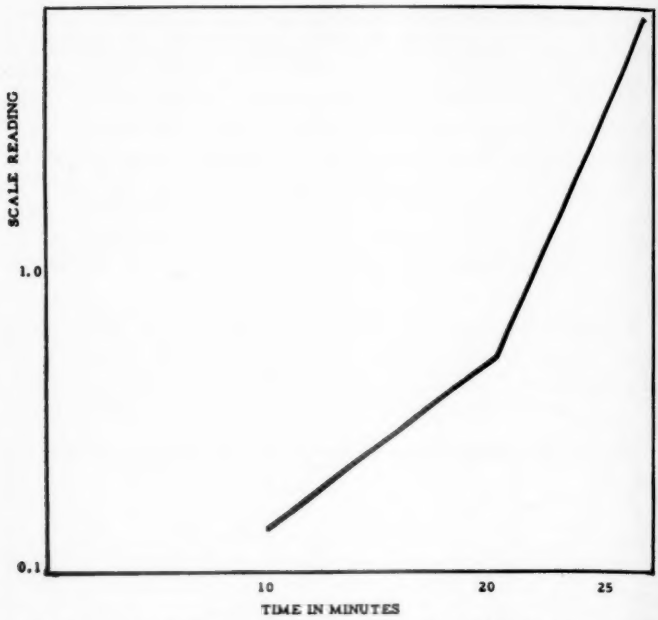

FIG. 6. The Rate of Progressive Immobilization of Gelatin Under the Influence of a Cross-Linking Agent (from Fig. 4) Plotted on Semi-Logarithmic Graph Paper.

mobilized is then fixed in the cell. It cannot perform its normal function but neither can it be excreted, so that it remains in the cell occupying space and as time goes by and as more and more of the proteins become cross-linked there is an increasing frozen metabolic pool which blocks normal function. (6–12)

This phenomenon can be observed directly in the unicellular organism Tokophrya Infusionum. It is well known that science owes much to a few organisms which show phenomena very clearly which in other organisms are obscured. Genetics owes much to Drosophila, with its clear-cut and easily manipulated genes, or to Ascaris, with its large chromosomes, and it may well be that in the future the Tokophrya organism will join this series. (40–44) In Tokophrya, the cell nucleus increases in size as the organism ages. It has a lifespan of about 12 days. As the nucleus expands, a chromatin body precipitates in the nucleus and this

precipitate increases with aging until the organism dies. If the lifespan of Tokophrya is shortened by overfeeding, this precipitation is increased in proportion so that the shortened lifespan corresponds to an accelerated visible precipitation of this chromatin material.

Zinsser (51) has furnished actual X-ray diffraction evidence of presence of cross-linkages in the human aorta protein with aging.

Other observations confirming that insolubilization of large molecules progresses with age are:

R. R. Kohn and E. Rollerson have shown reduction in swelling properties with age of human diaphragm tendon; (29) R. R. Kohn of perivascular lung tissue; (30) K. Kao, R. J. Boucek, and E. Noble of sponge biopsy connective tissue; (23) R. J. Boucek, N. L. Noble, and H. R. Eldon of collagen; (14) N. B. Medvedeva reported accumulation of insoluble protein material in heart, kidney, intestine, and lung; (33) Brown and Consden have shown a gradual rise in shrinkage temperature of human collagen with aging; (15) Bjorksten, Andrews, Bailey, and Trenk have found that tail tendons in senile rats show a 25% reduction in longitudinal heat shrinkage; (11) W. G. Banfield has shown reduction in swelling with age of the human Achilles tendon; (4) and Z. Dische, E. Borenfreund, and G. Zelmiens show insolubilization of lens protein of rats progressing with aging. (19)

The concept of the frozen metabolic pool in all aging cells is equally useful in explaining known clinical observations:

I. It is well established that diabetes generally accelerates aging phenomena, and diabetes is characterized by a ketogenic metabolism which gives rise to the type of organic substances which are known bi-functional substances and potential cross-linking agents.

II. It is well known that overfeeding shortens the lifespan much more than can be explained by the increased load on the heart. If a starving organism is fed some calories in one form or another, the combustion will go cleanly to carbon dioxide and water, with a minimum of time and a minimum of accumulation of intermediates. On the other hand, if an organism is overfed beyond the capacity of the enzymatic systems to utilize or digest, then there will be a backing up of the material and an

accumulation of intermediate products which are the type of products which are known potential cross-linkers.

III. It has been established that ionizing radiation shortens lifespan, even when applied in quantities insufficient to disturb the blood picture. (2, 3, 34, 48, 49) It is well known that ionizing radiation gives rise to free radicals which are powerful cross-linking agents, and they are used for this purpose in polymer chemistry, for instance, to increase the melting point of polyethylene (45). So here again we have a known effect, or a medium which is known to shorten the lifespan in a general way which cannot be pinpointed to any one cause, and which coincides with a known cross-linking effect.

The concept of a progressive trend of changes in protoplasm toward more stable, less reactive forms was clearly recognized by C. M. Child, who also takes full cognizance of the ability of organisms to effect various degrees of rejuvenescence by starvation periods, tending to force digestion of accumulated inert material (17). Child primarily attributes senescence to the accumulation of toxic substances, although he also mentions accretion of obviously inert substances such as fat, and refers to the possibility of still unrecognized factors.

He regards senescence as a necessary and inevitable feature of the life cycle, which can be balanced by rejuvenescence only in organisms low on the phylogenetic scale (18).

The more recent findings here presented justify a more positive view. Since the immobilization of large molecules such as protein has been shown with a very high degree of probability to be due to reaction between these molecules and some metabolites functioning as immobilizing agents, the death sentence resting upon all forms of higher life may become subject to reprieve. While human enzymes are incapable of re-mobilizing proteins or other high molecular polymers in which the points of attack of human enzymes have been blocked, the recognition of this mechanism in aging points the way for the search of enzymes or agents in low forms of life which might be isolated and used therapeutically as antigerics in humans. That enzymes capable of re-mobilizing inert proteins must be present for example in some soil bacteria is obvious from the absence of huge deposits of fossil protein.

A measure of the value of any theory is the fruitfulness of the

experiments which it suggests. If the theory which is advanced is correct, what should be done?

Obviously, the first thing will be to pinpoint the prime form of insolubilization of the proteins. This is a task for tracer technique and chromatographic technique. It will demand painstaking work, but nothing is involved in this task that could not be done with the proper application of time and of known techniques. When the form of insolubilization of the proteins is known, it will be possible to prepare *in vitro* a large amount of protein so cross-linked or so immobilized and use this as the sole nitrogen source in a culture of mixed soil bacteria and fungi. Any organism capable of growing in such a medium where the only nitrogen source is this immobilized protein, must have an enzyme which can split it. Then we can culture this organism, isolate the enzyme or agent in question, and use it in *in vivo* experiments to reverse the immobilization and unfreeze the immobile proteins. Naturally, it will be no easy task to find an agent, even by this technique, which can reach into the cells, maybe in the form of a precursor, and which is non-toxic, but the approach is logical and could well lead to substances which could be extremely powerful clinical tools (8, 13).

It is interesting also that this theory in no way conflicts with any fact known, but seems to be compatible with all observed clinical or experimental facts. The experimental work on the various facets of aging which is now going on has contributed enormously to our understanding. The time may now be ripe for joining together the many individual findings to a coherent picture and using this picture to design a master plan for a frontal attack to push back the limits of death due to aging to the same degree that has already been done with infant mortality and with infectious disease.

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PROCEEDINGS OF THE TEXAS BRANCH OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

Abstracts of Some of the Papers Read at the Spring Meeting

APRIL 1–2, 1960

THE UNIVERSITY OF TEXAS SOUTHWESTERN MEDICAL SCHOOL

DALLAS, TEXAS

Mast Cells and Anaphylaxis in Mice. R. D. Higginbotham, The University of Texas Medical Branch, Galveston, Texas.

The importance of mast cells to systemic anaphylaxis in the mouse is obscure. Although these cells contain both histamine and serotonin and can release these vasoactive amines in response to anaphylactic injury, the mouse is strongly resistant to the lethal effects of these amines as it also is to anaphylaxis. The effects of adrenalectomy and cortisol on resistance of mice to anaphylaxis, histamine and serotonin have been studied: 1) susceptibility to anaphylaxis increased more than 100 x after adrenex and could be partially restored by 250 μg cortisol, 2) histamine sensitivity (i.v. route) changed only slightly and was very resistant to cortisol prophylaxis (2500 μg), 3) serotonin sensitivity increased by 5 to 20 x in adrenex and resistance could be readily restored with cortisol (25 μg), 4) adrenalectomized mice were only slightly more sensitive to histamine and serotonin releaser drugs than intact mice. It is suggested that whereas mast cells may be of importance in local anaphylaxis, release of amines from this site would not seem to be sufficient to explain the lethal effects of systemic anaphylaxis in this species.

Ultramicroscopic Structure of *Bacillus anthracis* Growing *in vivo*. I. L. Roth, C. W. Lewis, Jr., and R. P. Williams, Department of Microbiology, Baylor University College of Medicine, Houston, and The University of Texas Electron Microscope Laboratory, Austin.

The electron micrographs demonstrated bacterial cells in relation to the host tissue. Bacteria in various stages of division were shown. Structures of the bacterial cells were well preserved, and included cell wall, cytoplasmic membrane, capsular residue, nuclear structures and prespores. Host cells showed well preserved mitochondria, nuclear and cytoplasmic membranes, and endoplasmic reticulum. A cytopathogenic area consistently appeared surrounding each bacterium. The fact that both bacterial cells and host tissue cells were well preserved made it possible to observe the deleterious effects of the bacterial infection upon the host cells. These effects were thought due to the exotoxin of the bacterium and ranged from the apparent disruption of cytoplasmic membranes to the complete dissolution of cell substance as observed in the areas designated as cytopathogenic.

Isolation from Egg-Yolk Dialysate of a Factor Stimulatory for the Growth of Mycobacteria.

James O. Norman and Robert P. Williams, Department of Microbiology, Baylor University, College of Medicine, Houston, Texas.

Continuous ether extraction of concentrated egg-yolk dialysate yields a yellowish, ether soluble fraction that is 70 times more active for growth of mycobacteria than crude dialysate. Color can be removed by charcoal filtration. The filtrate remains active. By paper chromatography the ether fraction can be separated into neutral fats, fatty acids, a phosphate fraction, and an unknown fraction. Only the latter stimulates mycobacterial growth. It is 240 times more active than crude dialysate. Although the active material has not yet been identified, it has properties similar to Coenzyme Q and menadione (vitamin K). However, the latter substances by themselves do not stimulate growth of our mycobacterial species. The egg-yolk material stimulates growth of *Mycobacterium tuberculosis* H37Ra and H37Rv as well as a scotochromogenic strain.

Intestinal Antisepsis: Comparative Studies with Neomycin and Neomycin-Nystatin.

A. W. Bronwell, M.D. and J. S. Hubbert, Lubbock, Texas.

Comparative studies, including microbiological evaluations, of the efficacy of neomycin-nystatin combination for preparation of the bowel prior to elective gastrointestinal surgery indicates that neomycin and nystatin together are preferable for simultaneous suppression of bacteria and fungi that normally co-exist in the intestinal tract.

Neomycin alone is satisfactory for prevention of bacterial proliferation but its efficacy as a bactericidal agent favors the outgrowth of yeast like organisms once a competitive environment is eliminated. The addition of nystatin to the antibiotic achieves a state of near sterility in a number of cases. Given for a period of 36 hours, neomycin-nystatin markedly reduced intestinal flora beginning on the third day and continuing through the sixth day. A slow return of the microbiologic spectrum to normal levels occurred on the seventh or eighth day following initiation of treatment. No untoward reactions to neomycin or neomycin and nystatin in combination were observed in a series of 21 patients treated in the course of this investigation.

The Isolation of Clostridium Species from Secondary Recovery and Salt Water Disposal Systems.

C. O. Tant, Visco Products Company, Inc., Houston, Texas.

The existence of bacterial corrosion of iron and steel by Desulfovibrio species has been established for many years. This study describes a method for the isolation from water injection systems of sulfide-producing Clostridium species of importance in corrosion. Anaerobic agar without dextrose (BBL) was modified by the addition of 0.1 gm/l of $Fe(NH_4)_2SO_4$ with the agar content

reduced to yield a semisolid medium. Presumptive identification of sulfide-producing clostridia was on the basis of black colonies and gas. The clostridia isolated were normal soil flora with the exception of several strains which could not be identified as previously described organisms. Studies of 400 water samples obtained throughout the United States strongly indicate the existence of possible relationships between clostridia, sulfate-reducers, and aerobic bacteria in the corrosion process.

Intracellular Localization of Pigment in *Serratia marcescens*.

Mira Purkayastha and Robert P. Williams, Department of Microbiology, Baylor University College of Medicine, Houston.

Bacterial cells were disrupted by sonic treatment, and the sonicated material fractionated by differential centrifugation. Hexosamine was liberated from the various fractions using lysozyme treatment. Pigment was extracted by an alkali-ether procedure. Both of the substances were determined quantitatively in a colorimeter. Results demonstrated that the content of liberated hexosamine and extracted pigment in the fractions paralleled each other. The maximum amount of both was in the 10,000 rpm fraction. Examination of this fraction in the electron microscope revealed the presence of typical cell envelopes. After hydrolysis no material absorbing at 260 mμ could be found in the fractions. These results indicate that the pigment of *Serratia marcescens* is located in the bacterial cell envelope. In addition, cell envelope material prepared by a chemical method also contained the maximum amount of both hexosamine and pigment.

Effect of Incubation Temperature and Nutrition Upon Pigmentation in *Serratia marcescens*.

Cora L. Gott and Robert P. Williams, Department of Microbiology, Baylor University College of Medicine, Houston.

Pigmentation occurs over a temperature range from 12 to 36°C in both complete and minimal media. Maximum pigmentation occurs at 28°C and drops off rapidly on either side. When followed for 7 days, pigmentation begins earlier in complete medium, but maximum values attained are identical for both media. Pigment production starts after the logarithmic phase of growth has ended, although protein synthesis is still occurring. Pigment continues to increase after the viable count and protein synthesis have declined. No pigmentation occurs at 38°C. However, cultures incubated at 38°C then brought to 27°C will pigment if incubated at 38°C no longer than 48 hr. in minimal, or 72 hr. in complete medium. Ability to pigment after the temperature is lowered appears due to an increase in viable cells.

Evidence of a Role for Soil Fungi in the Degradation of Cholesterol.

William J. Mandy, and G. E. Peterson, Department of Biology, University of Houston, Houston, Texas.

Cholesterol metabolism, while studied thoroughly in metazoan tissues, has received relatively little attention from microbiologists. Earlier researchers in this area indicated that actinomycetes and bacteria, but not molds, are involved in the catabolism of soil cholesterol.

We also noted that ten soil fungi failed to degrade cholesterol when the latter was used in a salts medium as the sole carbon source. However, when a growth promoting carbon source such as glucose or ethanol was added to the shaken culture medium, as much as 95% cholesterol utilization was noted after 7 days incubation. Up to 5.0 gm of cholesterol per liter of medium could be used by most of the molds. Controlled extractions showed very little binding of the sterol by the mycelium. Confirmation of Wintersteiner's conclusions on the auto-oxidation of cholesterol in heated aqueous suspensions indicated that the usual methods of preparing microbial culture media are inadequate for studies of this nature. A filter sterilized sterol solution with complete auto-oxidation controls must be used.

Spectrophotometric, chromatographic, and colorimetric tests of culture extracts failed to show any steroidal or bile acid degradation products of cholesterol. Hence it is likely that cholesterol utilization by these organisms involved a primary attack on the ring structure of the sterol. Studies with carbon 14 are planned in order to test this hypothesis.

A Fungal Pigment with Certain Unique Physical and Chemical Properties.

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Pigments with characteristics almost unique to the anthocyanins and anthocyanidins of flowering plants have not been demonstrated in fungi. Anthocyanins possess indicator properties which have not been described in the literature of known fungal pigments.

A pigment recovered from a submerged culture of one of our laboratory molds is red when acid and blue when basic. Pigmentation is markedly reduced when incubated in total darkness, and fails to pigment in broth containing organic nitrogen. Maximum pigmentation was obtained in a mineral medium containing 0.5% glucose.

This pigment was not soluble in those solvents commonly used for extraction of fungal pigments; extraction was best accomplished by using the same solvent system as that recommended for anthocyanin extraction; viz. n-amyl alcohol: acetophenone (2:1).

Spectrophotometric analysis of this pigment compared exactly with the published data of several anthocyanidins. Although the indicator properties and spectrophotometric analysis suggest an anthocyanidin type pigment, little

other evidence has been obtained to support this hypothesis. If a solvent suitable for molecular weight determinations can be found, this, followed by acetylation, identification of oxidation products, etc., may elucidate the true nature of this pigment.

Rapid Identification of Toxigenic *C. Diphtheriae.*

Jo Anne Whitaker, John D. Nelson and Chester W. Fink, Southwestern Medical School, Dallas.

The recent increase in incidence of diphtheria in the Dallas area stimulated the search for a rapid, reliable laboratory test for making a positive diagnosis of diphtheria.

The fluorescent antibody technique has been successfully used for identifying a variety of bacteria. Diphtheria antitoxin rather than bacterial antibody was conjugated with fluorescein isothiocyanate in the hope that this would differentiate between toxigenic and non-toxigenic *C. diphtheriae.*

Acetone-fixed throat swabs from seven bacteriologically proved cases of diphtheria seen in February 1960 were examined with the fluorescence microscope after staining with conjugated antitoxin. In all of the seven cases pleomorphic rods with bright fluorescent staining were observed. Specimens from 7 cases of non-diphtheritic membranous tonsilitis failed to show fluorescent organisms. Throat smears of six household contacts of one patient were examined by this technique and one young sibling was fluorescent-positive. Toxigenic *C. diphtheriae* was cultured from her throat.

Non-toxigenic *C. diphtheriae* exhibited no staining with the conjugated antitoxin nor did fifteen different organisms commonly found in the nasopharynx and gastrointestinal tract. Since this test appears specific and can be completed within an hour, this preliminary work indicates that the fluorescent antitoxin method may be a valuable adjunctive test for the diagnosis of diphtheria.

Effects of a Sulfated Polysaccharide on Resistance of Mice to the Pathogenic Effects of EMC Virus.

A. Y. Elliott and R. D. Higginbotham, The University of Texas Medical Branch, Galveston, Texas.

Carrageenans are a group of sulfated polysaccharides which have been used experimentally by others to induce alterations in connective tissue. The polysaccharides are rapidly accumulated by cells of the R.E.S., and it was of interest in the present study to determine whether this substance might alter the pathogenic characteristics of EMC virus infection in mice. Mice pretreated with 0.5 mg carrageenan by the i.p. route two hours prior to infection by this same route were found to have an enhanced resistance to the pathogenic effects of the virus infection with a survival of $54 \pm 0.5\%$ vs. 0% for controls. A neutral polysaccharide, Gelidium cartilagenium, also enhanced resistance, but to a lesser extent (30% survival). When the interval between treatment and infection was increased or decreased beyond the two-hour

period, the effect of treatment on resistance was only slightly evident if at all. From these and other experiments it appears that carrageenan may produce certain changes in mice which result in an alteration of the pathogenic effects of EMC virus infection on the host, but without markedly altering either dissemination or multiplication of the virus in the tissues of the host.

VIRULENCE OF STAPHYLOCOCCI IN MICE: A COMPARISON OF THREE METHODS OF CHALLENGE[a]

JOSEPH A. BASS AND R. D. HIGGINBOTHAM[b]

The resurgence of interest in the staphylococci which has resulted from hospital epidemics, has intensified efforts to elucidate virulence factors and immune mechanisms associated with staphylococcal disease. Investigations with these organisms are seriously hampered by the lack of a suitably susceptible laboratory animal. This situation is further complicated by variation in naturally occurring antibody titers among some laboratory animals, a fact which necessitates the use of large numbers of animals in any given experiment. Although the mouse appears to be no more highly susceptible to experimental staphylococcal infections than most other laboratory animals, its low cost and ease of handling offer obvious advantages over the use of larger animals.

A number of challenge routes and procedures have been employed in attempts to assess virulence of staphylococci for mice. For example, Christie, North and Parkin (1946), using heavy inocula by the intraperitoneal route, have related lethality to alpha toxin production. The more sensitive intravenous route has been employed by Gorrill (1951, 1958). Although not definitely stated, no clear-cut differences in virulence could apparently be demonstrated among coagulase positive staphylococci in studies involving a small number of strains. On the other hand, Smith and Dubos (1956) and Smith (1956), using a similar procedure, were able to detect differences in virulence among strains from various sources.

Selbie and Simon (1952) and Howard (1954) have attempted to assess virulence by inoculation of overnight-broth cultures intramuscularly into the thigh. Virulence has been related to the degree of swelling, and has been attributed to alpha toxin or to a combination of this and other exotoxins or antigens. The use

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of mucin to lower the resistance of the host when mice are challenged intraperitoneally has been widely used since its introduction by Nungester, *et al.*, in 1932, and has received application in experimental chemotherapy (Ercoli, *et al.*, 1945). More recently, the intracerebral route of inoculation has been suggested for testing efficacy of antibiotics against staphylococci *in vivo* (Panisset and Dobija, 1953), and virulence by this route has been shown to be due primarily to alpha toxin (Frappier and Sonea, 1956; Sonea, Frappier, and Borduas, 1956).

Although extensive studies have been carried out in some instances on the pathology and mechanism of death by a particular route, little or no attempt has been made to compare exotoxin production, time of death, etc., with results obtained by more than one method of challenge in the mouse. The present studies were therefore undertaken in an effort to compare intravenous and intraperitoneal routes of challenge, as well as intraperitoneal inoculation of organisms suspended in mucin. In addition to mouse virulence (as measured by lethality), exotoxin activity, phage type, and source of the organisms were considered.

Materials and Methods

Strains tested: Of the forty strains of staphylococci tested, 33 had been recently isolated from human infections. All of these isolates were coagulase positive, fermented mannitol, and produced varying gradations of yellow pigment. One strain (3416) had been isolated approximately one year earlier from the anterior nares of a patient, and was characterized by the production of a soluble yellow pigment (Bass and Verwey, 1960). The "Fritchie" strain, originally isolated from a granuloma, has been described previously (Higginbotham and Dougherty, 1955). The "Smith" strain, which was weakly coagulase positive and non-pigmented, was kindly furnished by Dr. Kay Miller of Merck, Sharp and Dohme Laboratories. The strain designated as "Wood" was from a departmental stock culture collection; strain Lederle 161 is also a subculture of the original Wood 46 strain. Strains designated as Lederle 161, 175, and 240 were kindly furnished by Dr. M. S. Cooper of the Lederle Laboratories. All strains were tested for susceptibility to antibiotics by the disc method, and were phage typed by the method of Blair and Carr (1953). Cultures employed in these tests were transferred to trypticase soy agar slants (BBL) from single colonies, and were placed under oil if they were to be held for more than a few days.

Preparation of suspensions: A trypticase soy agar slant was heavily inoculated from the stock slant and incubated at 37C overnight. A heavy saline suspension was prepared from the fresh slant and a drop of this inoculated into 100 ml brain heart infusion broth in a 250 ml Erlenmeyer flask. Following

3 to 4 hours' incubation at 37C, the cells were harvested by centrifugation at high speed (approximately 10,000 rpm for fifteen minutes). Organisms were then resuspended in M/15 phosphate buffer pH 7.4, to the desired turbidity as determined with a Klett-Summerson Colorimeter, using a 540 mu green filter.

Inoculation procedures: In all instances, 0.25 ml of a suspension of organisms equivalent to approximately 1 x 10^8 viable cells was used as the challenge dose. For intraperitoneal and intravenous challenge, the organisms were suspended in M/15 phosphate buffer. For intraperitoneal inoculation with mucin, a two-fold concentration of organisms was mixed with equal amounts of 10% mucin adjusted to a pH of 7.4. The same lot of mucin (Armour Control No. H 32002) was used throughout. Control animals receiving mucin only were included in all tests.

Test animals: 21–25 gram female white mice, CFW strain, obtained from Euers Farm, Austin, were employed in all tests. Mice when received weighed 15 to 20 grams, and were allowed to acclimate for approximately one week before use. Test animals were observed for a total of 21 days after challenge, but since little change occurred in results following the tenth day, results cited are those observed at the end of ten days' observation.

Tests for hemolysin production: For alpha hemolysin production, a modification of Casman's semisolid agar method (Casman, 1940) was followed. The formula used consisted of:

Veal infusion broth (Difco)	25 grams
Sodium acetate	7 grams
Proteose peptone # 3 (Difco)	10 grams
Noble agar (Difco)	5 grams
Distilled water	1000 ml

0.1 ml of an overnight brain heart infusion broth culture of the organism was inoculated onto the surface of 25 ml of semisolid agar in a 250 ml Erlenmeyer flask. The flask was then thoroughly flushed with oxygen and a volume of CO_2 equal to approximately 40% of the atmosphere subsequently added by displacement. Flasks were incubated at 37C for 72 hrs. without renewal of the gaseous environment. Flasks were then placed at —20C until the agar was thoroughly frozen, then removed to room temperature to allow agar to thaw. The agar was scraped into plastic tubes and centrifuged at approximately 10,000 rpm for 15 minutes. Supernates were kept at —20C until used for alpha toxin determinations. Lh units were determined in the usual manner; rabbit antitoxin which had been standardized against an international unit was used as a reference. Duplicate tests with several cultures on different occasions gave good agreement.

Trypticase soy agar containing 2% washed sheep red blood cells was used for determining beta hemolysin production. Duplicate plates were incubated in regular atmosphere and under anaerobic conditions, followed by refrigeration overnight. Anaerobic incubation was used in an attempt to reveal small amounts of beta hemolysin which might be masked by alpha or delta lysins. Although the latter toxins were produced in only small quantities anaerobically, beta hemolysin was also markedly reduced. No instances of

production of beta lysin were noted which were not detectable under aerobic conditions.

For detection of delta hemolysin production, measured amounts (10 ml) of trypticase soy agar, containing 2% washed red blood cells, were poured into flat bottom Petri plates. The strains were stab inoculated with a straight needle from young broth cultures onto two each of human and horse blood agar plates. One set of plates was incubated in regular atmosphere, the other in an atmosphere of approximately 40% CO_2 and 60% O_2. Contrary to the findings of other workers (Marks and Vaughan, 1950), zones produced under increased CO_2 concentration were smaller than those under regular atmosphere. The diameter of the colony was subtracted from the diameter of the zone of hemolysis after the method of Marks and Vaughan (1950). The findings reported herein are a sum of the diameters produced on the four plates (horse and human blood, with and without CO_2).

Results

Table 1 summarizes the findings of all experiments. It may be readily seen that no correlation exists between antibiotic resistance or sensitivity of staphylococci and their ability to produce death by any of the three methods employed. Consideration of phage types might lead to the conclusion that non-typables and those typable only with the HJD 44A phage tend to be highly virulent by two of the three methods employed, whereas other phage types appear to be virulent primarily by only one procedure. However, the fact that a large number of the non-typable strains were blood isolates might tend to prejudice these findings (*vide infra*).

With the exception of strains typable with 80 and 81 phages, no other types were present in sufficient number to permit evaluation of a given phage type. Examination of the 80/81 strains shows this group of 12 organisms to be poorly pathogenic by the intravenous route (median of less than 30%, with a range of 0 to 50%) as opposed to a fairly high virulence by this route exhibited by the other 21 freshly isolated strains (median 60%; range 0–100%). All groups were relatively avirulent by the intraperitoneal route with the dosage employed (median less than 10%), but the inclusion of mucin raised mortality due to the 80/81 strains to a median of 70%. By way of comparison, suspension in mucin raised mortality of the other strains to 60%, the same per cent mortality which was obtained with the organisms by the intravenous route. Both 80/81 strains and the remaining types produced alpha hemolysin to approximately the

TABLE 1

Virulence of Staphylococci for Mice: Comparison of Various Phage Types

Strain number	Phage pattern	Source	Antibiotic resistance pattern†	Exotoxins			Virulence (Deaths/10)		
				Alpha (Lh/ml)	Beta	Delta‡	I.V.	I.P.	I.P. Mucin
675	NT	Blood	PT	10	+	22.5	8	0	7
959	NT	Blood	PT	3		21.5	10	0	6
1098	NT	Paronychia	PTE	6		13	4	0	8
3416	NT	Nose	PTEO	<1	+	21	10	0	7
1578	NT	Blood	PT	21	+	24	10	0	7
1610	NT	Blood	P	13	+	25.5	9	1	4
1668	NT	Blood	PT	13		8	5	0	6
1476	44A*	Carbuncle	PTE	6		11.5	2	0	8
970	44A*	Abscess	P	13		19	8	0	7
1972	44A*	Blood	(CS)	3		10.5	6	0	6
Fritchie	44A*	Granuloma	(CS)	2		3.5	1	10	10
Smith	44A*	(Lab Strain)	(CS)	<1	+	Trace	1	10	9
519	52/44A*	Wound	PTE	7		11.5	2	0	10
1923	3B/44A*	Blood	T	<1	+	2	8	0	5
1922	3B/44A*	Blood	(CS)	<1	+	Trace	9	1	8
2241	77/44A*	Blood	T	16		16.5	10	0	5
372	39/44A*	Blood	(CS)	6		20.5	2	1	8
Wood	42D/44A*	(Lab Strain)	(CS)	12.5		17	1	0	4
Lederle	42D/44A*	(Lab Strain)	(CS)	>50		17.5	1	0	5
161 (Wood) Lederle 240	42D/44A*	(Lab Strain)	(CS)	63	+	5.5	6	2	10

618	3B/3C/39/55	Furuncle	B	11	20.5	3	0	6
485	3B/3C/39/55	Boil	(CS)	2	19.5	3	0	2
Lederle 175	3B/3C/39/44A*	(Lab Strain)	(CS)	2	6.5	0	0	3
815	39/55	Furuncle	B	2	19	1	0	3
542	7/54/73	Wound	P	4	18.5	7	0	5
2205	77/44A*	Blood	T	3	18	10	0	8
1258	52/80	Wound	PTE	2	Trace	0	0	0
785	52/80	Wound	PTEO	3	Trace	1	0	1
701	42B/80/81	Blood	P	17	12.5	1	0	7
2148	42B/52/80/81	Wound	PT	6	12.5	0	0	0
346	42B/52/80/81	Wound	PTE	5	11.5	3	3	7
347	42B/52/80/81	Burn	PT	5	11	1	2	6
843	42B/52/80/81	Abd. Pustule	PTE	2	5.5	2	0	2
1123	42B/52/80/81	Abscess	P	<1	10.5	3	0	10
636	42B/52/80/81	Wound	PTK	6	12	1	1	9
FB	42B/52/80/81	Boil	PT	6	11.5	4	0	9
180	42B/52/80/81	Blood	PT	5	14.5	4	0	8
1097	42B/52/80/81	Boil	PTE	6	9	5	0	6
1458	42B/52/80/81/44A*	Boil	PT	6	12.5	3	0	10
1572	42B/52/80/81/44A*	Abscess	(CS)	<1	5.5	1	0	5

* Lysed by HJD 44A phage only.
† P, penicillin; T, tetracycline; E, erythromycin; O, Oleandomycin; B, bacitracin; (CS) sensitive to all antibiotics tested.
‡ Sum of diameters in mm. of zones of hemolysis on human and horse blood agar plates.

same extent, with values ranging from less than 1 Lh unit/mL to as high as 21. Delta toxin, while somewhat lower for 80/81 strains than for a number of other phage types, was nonetheless more consistently produced in this group. It is of interest to note that one strain (2148) having a phage pattern and other *in vitro* characteristics of the "hospital" strain, failed to produce any deaths by any of the three methods of challenge. Similarly it should be noted that the variation in mortality obtained with this group of organisms indicates that mouse virulence testing under the present conditions would not serve to differentiate potentially troublesome strains from others having similar *in vitro* characteristics. It is further apparent, from a consideration of all the strains tested, that the evaluation of virulence by only one method is insufficient, since a given strain may be highly virulent when tested by one method but not by another. This, of course, suggests that different virulence factors are being measured by the three methods.

However, in examining the source of the organisms, it was noted that the large majority of those strains isolated from bloodstream infections were highly virulent by the intravenous route. Thus the median lethality of this group intravenously was 80% as opposed to 30% for other freshly isolated strains, yet the production of alpha and delta toxin was not significantly greater (medians of 6 and 5 Lh/ml, respectively). Zones of delta lysis appeared to be greater with the blood isolates group, but not significantly so. The production of beta toxin by 5 of the 13 strains may be of significance, since this toxin is more commonly associated with animal pathogens. However, the high mortality rates obtained with other strains not producing this lysin would indicate that its role may well be coincidental or perhaps secondary.

Table 2 compares those strains demonstrating high I.V. virulence to those with low virulence by this route. As with the blood isolates, no significant differences in alpha toxin activity was noted, and only questionably higher delta toxin production. The occurrence of beta toxin in 7 of 15 strains might suggest that a combination of toxins is responsible for higher I.V. virulence; however, several strains with only moderate or poor toxin producing capacities (e.g., 959, 1923, 1922, 2205) demonstrate a high degree of virulence (greater than 50%) by both intravenous and intraperitoneal-mucin methods. Conversely, three

TABLE 2

Comparison of characteristics of strains of Staphylococcus aureus *highly virulent intravenously to those strains demonstrating low-grade virulence by the same route*

	High (50% or greater mortality)	Low (less than 50% mortality)
No. of strains	15	25
Median percent Mortality I.V.	80 (50–100%)	10 (0–40%)
Median percent mortality I.P. + Mucin	60 (40–100%)	70 (0–100%)
Median alpha toxin (Lh units/ml.)	6 (<1–63)	5 (<1–50)
Median delta toxin (Sum of dia. of zones in mm.)	18 (0–25.5)	11.5 (0–20.5)
Beta toxin produced by:	7/15	1/25

strains producing substantial alpha toxin *in vitro* (1458, 618, 636) do not exhibit high intravenous lethality, and two strains (2148, Wood) show poor virulence by all three methods. It should again be noted that the inclusion of mucin increases mortality sharply when poorly I.V. virulent strains were tested intraperitoneally, but actually fails to raise mortality of highly I.V. virulent group above the level of virulence achieved by intravenous challenge alone.

If one examines those strains whose lethality was significantly "enhanced" when injected by the peritoneal route in the presence of mucin (40% or greater increase as compared to lethality obtained with these same organisms injected alone), it is seen (Table 3) that there is no significant difference in the two groups regarding beta or delta toxin production. The difference in alpha toxin production may be of significance; however, seven strains showing poor alpha activity were substantially enhanced by mucin (strains 1123, 1572, 2205, Fritchie, 3416, 1923, 1922). Neither could lethal effects be accounted for by a combination of toxins, since three of these seven strains exhibited very poor overall toxic activity.

Table 4 summarizes the exotoxin activity of strains either

TABLE 3

Comparison of characteristics of strains of Staphylococcus aureus *highly lethal when injected intraperitoneally with mucin to strains poorly lethal by the same route without mucin*

	(High (lethality increased 40% or more)	(Low (lethality increased less than 40%)
No. of strains	31*	8
Median percent mortality I.P.	<10 (0–100%)	<10 (0–30%)
Median percent mortality I.P. + Mucin	70 (40–100%)	20 (0–40%)
Median alpha toxin (Lh units/ml.)	6 (<1–63)	2 (2–13)
Median delta toxin (Sum of dia. of zones in mm.)	12.5 (0–24)	11.5 (0–25.5)
Beta toxin produced by:	6/31	1/8

* Smith strain not included, since experiments have not been conducted to determine whether smaller numbers are required to produce 100% death in the presence of mucin than in its absence.

highly virulent by one or more methods or poorly virulent by all three. The complete lack of consistency in the production of any one toxin or of a combination of toxins by any one group is readily evident. These findings indicate that although exotoxins are of importance in determining the outcome of infection, the factor or factors which precipitate or initiate the pathogenic process might be attributable to some other characteristic.

For comparison of the "mucin enhanced virulence with virulence by intraperitoneal challenge alone as well as by intravenous challenge, the results were plotted as shown in Figures 1 and 2. Although 31 of the 40 strains were avirulent when injected intraperitoneally alone (Fig. 1), subjection of the host to the resistance lowering effects of mucin increased lethality of the large majority of the strains to 50% or greater. The Fritchie strain, which is highly virulent intraperitoneally alone, is lethal in relatively small numbers when injected with even small amounts of mucin (Higginbotham and Bass, 1960a). The original Smith strain is similarly enhanced (Verwey, 1960); however, the strain used in the present experiments, although possessing the usual intraperitoneal virulence characteristics of the Smith strain, is non-pigmented and may behave differently.

In most instances (Fig. 2) mucin also enhanced lethality as opposed to virulence of the same organisms by the intravenous route. As noted previously, however, mucin failed to raise I.P. mortality of highly I.V. virulent organisms to the level of virulence achieved by intravenous challenge alone. On the other hand, a large number of strains which produced less than 50% deaths intravenously were highly lethal when injected with mucin intraperitoneally.

If the two methods of challenge had given approximately the same mortality values, the majority of the points in Figure 2 would have fallen on or near the diagonal line. The fact that few of these points are located on or near this line suggests that deaths by the two methods (intravenously, and intraperitoneally with mucin) were due to different mechanisms. This difference in

TABLE 4

Comparison of Exotoxin activity of strains of Staphylococci highly virulent by one of more methods of challenge to that of strains poorly virulent by the three methods used

	Virulence		*Exotoxins*		
I.V.	I.P.	I.P. mucin	Alpha (Lh u/ml)	Beta	Delta (Sum of dia. of zones in mm.)
10	0	6	3		21.5
10	0	5	16		16.5
10	0	7	<1	+	21
10	0	8	3		18
10	0	7	21	+	24
1	10	10*	2		3.5
1	10	9	<1	+	Trace
3	0	10	<1		10.5
3	0	10	6		12.5
6	2	10	63	+	5.5
2	0	10	7		11.5
0	0	0	2		0
0	0	0	6		12.5
2	0	2	2		5.5
1	0	3	2		19
1	0	4	13		17
1	0	1	3		Trace
3	0	2	2		19.5
0	0	3	2		6.5

* Marked mucin effect.

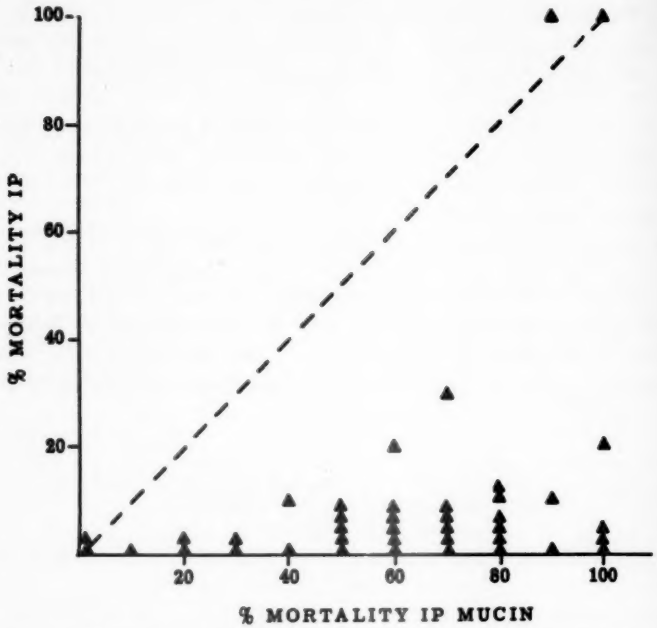

FIG. 1. Comparison of mortality produced by strains of *Staphylococcus aureus* when injected by the intraperitoneal route to that obtained when suspended in mucin.

lethality is further emphasized by the fact that the median time of death resulting from intraperitoneal-mucin challenge is less than 24 hours (83% within 24 hours; 95% within 48 hours), whereas median time of death by the intravenous route is 5 days.

Discussion

From the present studies, it is not possible to determine what mechanisms are involved in the lethality of staphylococci for mice by the three methods of challenge employed. Although alpha toxin has been correlated to lethality when large numbers of organisms are injected intraperitoneally (Christie, North, and Parkin, 1946) and to the degree of swelling following intramuscular inoculation (Selbie and Simon, 1952), the present findings do not correlate high degree of lethality by any of the three routes

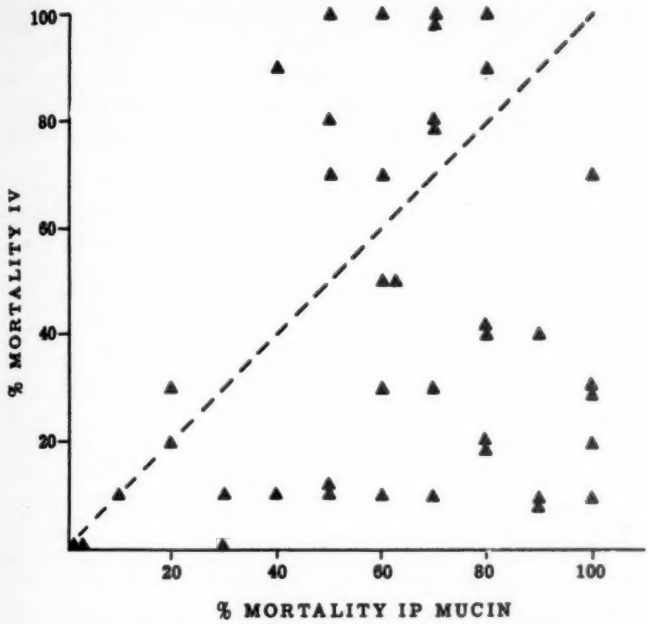

FIG. 2. Comparison of mortality produced by strains of *Staphylococcus aureus* when injected intravenously to that obtained when injected intraperitoneally in the presence of mucin.

with the *in vitro* production of this toxin. Although its presence singly or in combination with other exotoxins has been shown by Selbie and Simon (1952) and by Howard (1954) to be related to the degree of swelling following intramuscular challenge, the same conclusions could not be drawn with regard to lethality under the present conditions.

Inasmuch as all strains tested were coagulase positive, with the possible exception of the Smith strain, it was obvious that marked differences in virulence as measured by lethality can occur amongst coagulase positive organisms recently isolated from human infections. That gradations in virulence can be detected is apparent. Unfortunately, the present methods do not distinguish between strains with higher potential for human infection such as the 80/81 strains and other strains less commonly associated with recent hospital epidemics. On the other hand, the find-

ings obtained with strains from blood stream infections strongly suggest that such strains are in general more highly virulent for mice by the intravenous route. If the bacteremic state is taken as a reflection of the seriousness of a human involvement, then virulence of staphylococci for mice might well be correlated with virulence for humans, as suggested by Smith (1956).

It is well to keep in mind that the elements responsible for virulence in different animal species can vary widely (Elek, 1959), and conclusions drawn from these and similar experiments cannot be freely applied to man. Nonetheless, it is an incontrovertible fact that *in vivo* experimentation will be necessary for further elucidation of the pathogenesis of staphylococcal infections, since in spite of the many *in vitro* characteristics associated with potentially pathogenic staphylococci, no clear-cut relationship can be established to the occurrence of any single factor or group of factors as demonstrated *in vitro*. It is therefore of prime importance to determine whether results of studies on the same species challenged in different ways are the result of the same or different factors.

It would appear from the present findings that this is not the case, since intraperitoneal challenge with mucin "enhances" virulence of some strains completely out of proportion to that exhibited by the intravenous route, and conversely does not "enhance" lethality to an equal degee in those that are highly pathogenic intravenously. The fact that the median time of death in the mucin-treated animal is less than 24 hours, as opposed to a median time of death of 5 days in the intravenous challenged group further emphasizes these differences. In addition, the only two strains highly virulent by the intraperitoneal route without mucin were almost completely avirulent intravenously. These data indicate strongly that death by these two routes are due to different factors and that factors enhanced by mucin may be vastly different from those responsible for intravenous virulence (Elek, 1959; Bass and Higginbotham, 1960b).

Since high intravenous lethality cannot be correlated closely to alpha, beta or delta toxins, or to a combination of these, it would be of interest to determine whether those organisms demonstrating a high degree of virulence by this method might possess singular "nidogenic" characteristics which enable them to localize in the tissues and subsequently cause death of the host.

Similarly, in view of the lack of correlation of exotoxin activity to high mucin-intraperitoneal lethality, additional factors must be examined. Studies to be reported on elsewhere strongly indicate that an endotoxic-like substance produced by staphylococci is probably involved (Higginbotham and Bass, 1960b).

Summary

Groups of mice were challenged by three different methods (intraperitoneally, intravenously, and intraperitoneally with organisms suspended in mucin) with the same volume of a standardized suspension of viable cells of *Staphylococcus aureus*. Forty strains were tested, 33 of which were fresh isolates from human infections. Results indicated that virulence of staphylococci for mice (as measured by lethality) could not be correlated to antibiotic sensitivity or resistance or to phage type, including 80/81 strains typical of those involved in hospital epidemics. On the other hand, strains isolated from blood stream infections demonstrated greater virulence when injected by the intravenous route than did other strains. However, it was apparent that evaluation of virulence by only one method was insufficient, since a given strain might be highly virulent by one route but not by another.

Virulence by any one method could not be correlated with exotoxin production. In general, the use of mucin to lower resistance of the host animal challenged by the intraperitoneal route markedly increased mortality over the mortality obtained intravenously. Furthermore, the median time of death with the two methods differed significantly. These data indicate that virulence by these two routes are due to different factors and that factors enhanced by mucin may be vastly different from those responsible for intravenous virulence.

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CHEMOTHERAPY OF EXPERIMENTAL HISTOPLASMOSIS[a]

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It is generally agreed that a need exists for a more effective drug for the treatment of histoplasmosis as well as for other deep mycotic infections. It has been estimated that probably 30 million persons in central United States have been infected with *Histoplasma capsulatum*.[11] The usual infection is limited to a pulmonary infection which is subclinical, but in some individuals the fungus spreads systemically where it multiplies intracellularly. Therefore drugs effective in the treatment of the severe systemic form of histoplasmosis would of necessity have the property of cellular penetration.

With this in mind, our attention was drawn to the area of drugs used for the treatment of malaria. The plasmodium of malaria is an intracellular parasite in all phases of its life cycle so that drugs must penetrate intracellularly to be effective. Many hundreds of compounds have been surveyed for an antimalarial effect during the wartime research program of screening under the Office of Scientific Research and Development[20] and some were available to us for these studies. We were particularly interested in drugs known to have a curative or prophylactic effect on malaria. Such an effect by a drug in either bird or primate malaria would indicate an activity on tissue stages residing in cells of the lymphoid-macrophage system in the case of bird malaria, or in the liver parenchyma cells in the case of primate malaria. In either event, a drug with activity in tissues might be effective in the treatment of disseminated histoplasmosis where the organisms are located chiefly in the cells of the lymphoid-macrophage system and particularly in the liver and spleen. In addition to the antimalarials, related drugs which are amebicidal, antibiotics with *in vitro* effect on fungi and miscellaneous drugs reported by other workers to have fungicidal properties were studied.

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Materials and Methods

In vitro methods

A strain of *H. capsulatum* obtained from Dr. N. F. Conant of Duke University in 1949 was used in all experiments unless otherwise specified. The majority of the drugs were originally tested in Bacto-brain heart infusion agar, pH 7.4 with 10 per cent of human blood. Sterilized solutions of the drugs were incorporated into the agar by the pour plate technique. A uniform inoculum was prepared from 48 hour yeast phase cultures, twice washed and suspended in sterile saline to contain approximately 100,000 cells per ml. by the nephelometer method. Excess moisture was removed and the plates inoculated by radial streaks with sterile cotton tipped swabs dipped into the inoculum. The plates were sealed and incubated at 37°C for 5 days. Control plates were inoculated at the same time for comparison of growth.

Mycophil broth (BBL) pH 7 was also used to assay the antibiotics for their effect on the growth of *H. capsulatum*. Sterile solutions of drugs were added to double strength broth and the total volume brought to 5 ml. with sterile distilled water. The inoculum was 0.1 ml. of a 48 hour yeast phase culture of *H. capsulatum* grown in Mycophil broth. Observations were made for growth at weekly intervals for 4 weeks and compared with control tubes containing no drug. A re-check of drugs found to inhibit growth by use of the agar dilution method showed that the results obtained with the Mycophil broth were quite similar.

Weighed quantities of various compounds were dissolved in water or ethanol and sterilized, either by autoclaving at 10 pounds pressure for 10 minutes, or in more instances by Seitz filtration when the drug was not heat stable. Where ethanol was used as a solvent for the drug, ethanol controls were included.

In vivo methods

A standardized experimental infection in white mice was employed for testing drugs *in vivo* against histoplasmosis. The mice used were all young females averaging 20 grams in weight and were CF1 strain from Carworth Farms. Yeast phase organisms from 72 hour cultures on Bacto-brain heart infusion agar with 10–20 per cent human blood added were washed twice in physiological saline and the volume of packed organisms obtained by centrifugation for 15 minutes at 1600 r.p.m. was diluted with physiological saline. As a result of preliminary trials, a 1:160 dilution of packed organisms was chosen as the best inoculum for testing drugs and each mouse was inoculated in the tail vein with 0.2 ml. of this dilution. Control mice were always inoculated at the same time as were the mice to be treated so that chance variation in the virulence of the infecting dose could be taken into account. The mortality achieved in untreated mice by this inoculum can be seen by scanning the columns for mortality and average day of death of untreated animals in tables giving the results of the various experiments. Uninfected drug controls were included in the experiment when maximum tolerated doses of drugs were administered. Drugs were administered as noted in the tables. Amounts of drug are given as the base unless otherwise specified. Touch smears were made of the spleen and liver of all mice which died within 4 weeks. These were stained with Giemsa and examined for *H. capsulatum*.

Four weeks after inoculation, survivors were sacrificed and their spleens and livers were cultured on Sabouraud's medium for information on eradication of the organisms.

The *in vivo* test described above is a relatively simple and short term procedure since mortality in untreated controls approached 100 per cent in little over a week. Both a decrease in mortality and an increase in average survival time can be used as indications of a drug's activity. The disease produced in mice by this method would simulate the disseminated disease in man rather than the pulmonary form of the disease. More drugs were tested in the experimental infection than in the test tube as our *in vivo* test was of short duration and could conceivably pick up drugs which are inactive *in vitro*. This activity *in vivo* only has been shown for the sulfonamides with experimental histoplasma infections in mice by Mayer *et al.*[12] *In vivo* testing would also differentiate drugs which are changed in the body to active or inactive metabolites.

Results and Discussion

The results of *in vitro* testing are listed in Table 1. Results of *in vivo* tests are grouped according to the type of compound and discussed below.

Acridines. The acridines, quinacrine and acranil, were chosen for *in vivo* testing because of the known tendency of quinacrine to concentrate in tissues, especially in the liver and spleen. However, as seen in Table 2, maximum tolerated doses in the diet of infected mice did not protect them against death. Campbell and Saslaw,[4] were able to show some beneficial effect with quinacrine on the experimental disease but this was when both organisms and drug were given intraperitoneally.

Quinolines. The iodo-hydroxyquinolines, Vioform, Diodoquin and chiniofon, were tested *in vivo* because of the low concentration of Vioform necessary for complete inhibition of *H. capsulatum in vitro* (Table 1). Furthermore, it has been shown by Haskins *et al.*[7] that an appreciable absorption of iodine results from oral administration of these drugs indicating absorption of the drug in some form by this route. Serum neutralization of the drug[1] is a possible explanation of the negative results shown in Table 2 of the *in vivo* testing with this group of compounds.

Primaquine was tested in the experimental infection because it is the most effective of the 8-amino-quinolines against the tissue stages of human malaria. Although the closely related 8-amino-quinolines, pamaquine and pentaquine were not inhibitory to the fungus in low concentration *in vitro*, metabolites of

TABLE 1

In vitro *effects of various drugs on the growth of the yeast phase of* Histoplasma capsulatum.

Drug group	Name of drug (salt)	Concentration giving complete inhibition of growth; μg/ml. base
Antibiotics	Chlortetracycline	>1000
	Chloramphenicol	>1000
	Streptomycin (sulfate)	>1000
	Neomycin (sulfate)	800
	Endomycin	2
	Thiolutin	2
	Rimocidin	1
Acridines	Rivanol (lactate)	>40
	Quinacrine (dihydrochloride)	20
	Acranil (dihydrochloride)	20
	Neo-Acranil	20
	Acridine Orange	20
	Acriflavine	3
Quinolines	Chiniofon	800
	Pentaquine (monophosphate)	750
	Pamaquine (hydro-iodide)	200
	Quinine (hydrochloride)	400
	Chloroquine (diphosphate)	2500
	Vioform	1
Miscellaneous	Para-amino-salicylic acid	5000
	Promin	3000
	Chlorguanide (monohydrochloride)	800
	8-(3-isopropyl-amino) 6-methoxylepidine (dihydrochloride)	400
	Metachloridine	300

these drugs have been demonstrated to have a greater antimalarial activity than the original compounds[10] so that they are more active *in vivo* than *in vitro*. The results in Table 2 show no beneficial effect of primaquine on the course of the disease, even with maximum tolerated doses.

Miscellaneous drugs: Biallylamicol is a drug which has a suppressive, but not a curative effect on human and bird malaria. However, following oral administration in animals, high concentrations of the unchanged drug are stored in the liver, lungs,

TABLE 2

In vivo results of treatment with acridines, quinolines, and biallyamicol

Drug	Administration: Method	Administration: Period (days)*	No. dead/total mice: Treated	No. dead/total mice: Untreated	Avg. day* of death: Treated	Avg. day* of death: Untreated
Acridines:						
Quinacrine	0.2% in diet	0 to death	5/5	5/5	9	13
Acranil	0.2% in diet	0 to death	5/5	5/5	9	13
Quinolines:						
Primaquine	0.03% in diet	0 to death	10/10	10/10	6.6	8.4
	0.09% in diet	0 to death	10/10	10/10	4.7	8.4
Vioform	0.25% in diet	−7 to +7	6/10	4/10	12.0	8.2
	0.25% in diet	−3 to death	10/10	10/10	6.9	9.1
Diodoquin	0.25% in diet	−7 to +7	9/9	4/10	8.6	8.2
Chiniofon	0.25% in diet	−7 to +7	5/10	4/10	15.0	8.2
Miscellaneous:						
Biallylamicol	0.02% in diet	−2 to 10	10/10	9/10	12.9	12.1
	0.06% in diet	−2 to 10	8/9	9/10	10.1	12.1
	0.18% in diet	−2 to 10	10/10	9/10	9.2	12.1

* Day of inoculation = 0

and various other tissues for relatively long periods. The drug has been shown to be active against amebic hepatitis in hamsters.[18] Repeated tests, summarized in Table 2, showed the drug to be without effect on histoplasmosis even when administered in maximum tolerated doses.

At the time our experiments were initiated, ethyl vanillate was the drug in current usage for treatment of histoplasmosis.[5] Preliminary trials indicated that a dose approximating that recommended for man (1.7 gm./K) had no beneficial effect on the experimental infection in mice. *In vitro* tests indicated that our strain of *H. capsulatum* was inhibited by 30 mg. per cent whereas the human dose was intended to result in blood levels of approximately 20 mg. per cent of ethyl vanillate. Therefore, we turned our attention to blood levels attained in mice with various doses of ethyl vanillate. Using the method of blood level determination published by Christie *et al.*,[5] it was found that administration of comparable amounts of the drug by gavage in Sorlate (sorethitan monooleate, Abbott) resulted in higher blood levels than the administration in either olive oil or in a drug-diet mixture. It further appeared that the highest dose which could safely be given in Sorlate was 4 grams per kilogram daily. A series of 10 mice given this dose in Sorlate by gavage b.i.d. for nine days all survived with no weight loss as a group. As is the case with human subjects the blood levels attained by individual mice given this dosage were quite variable. However, 29 per cent of 24 mice given 4 grams of ethyl vanillate per kilo daily in Sorlate were found to have blood levels of 30 mg. per cent or above, while 46 per cent of the mice had levels of 20 mg. per cent or above. When mice given our standardized infection were treated immediately after infection until death with such a dosage, the results shown in Table 3 were obtained. The only demonstrable effect of the drug was to hasten the death of the mice with histoplasmosis. Touch smears of treated mice made at autopsy showed plentiful organisms, indicating multiplication in spite of treatment. Thus, we were unable to demonstrate any beneficial effect of ethyl vanillate on disseminated histoplasmosis in the white mouse, confirming the reports by Saslaw and MacMillan[16] and Procknow and Loosli.[13]

Antimetabolites: The 2–4 diaminopyrimidines included in Table 4 adversely affected the course of the disease, decreasing

TABLE 3

In vivo *results of treatment with ethyl vanillate*

perimental group	Method of drug administration	Daily dose per kilo	Period of administration	No. dead/ total mice	Average day of death
fected; treated	52 mg. in 0.1 ml. of Sorlate b.i.d. by gavage	4–4.4 gm.	day 0 to death	10/10	2.8
fected; untreated	—	—	—	9/10	9
ninfected; treated	52 mg. in 0.1 ml. of Sorlate b.i.d. by gavage	3.8–4.3 gm.	7 days*	0/5	—

* Drug controls killed 2–2.5 hours after last dose. Blood levels were 6, 7, 12.5, 8.5, and 47.0 mg. per cent.

survival time and increasing mortality of mice so treated. Non-infected mice given the same amount of drug as infected mice gained weight. One of these drugs, pyrimethamine, is well known for its suppressive effect on blood stages of malaria and is used in combination with the sulfa drugs in the treatment of *Toxoplasma* infections. Drugs of this group are folic and folinic acid antagonists.

The heptyl and hexyl esters of p-hydroxybenzoic acid were tested because of a report[9] of their *in vitro* activity against a number of fungi, including *H. capsulatum*. These drugs, as shown in Table 4, were neither active nor toxic in high concentrations in the diet, raising a question as to their absorption upon oral administration.

Para-aminobenzoic acid (PABA) and its sodium salt were tested several times because an early test indicated that the drug had a protective effect. This observation could not be repeated (Table 4), and seems unlikely in view of the fact that the sulfonamides, which are PABA antagonists are now known to be protective against the experimental infection.[12]

It was hoped that metachloridine, a substituted meta isomer of sulfanilamide, might have some *in vivo* effect since concentrations of 30 mg. per cent are obtainable in the sera of rats fed 0.5 per cent of the drug in the diet.[20] This was also the concentration which was found to inhibit the organism *in vitro*. As shown in Table 4, mice given an amount by gavage approximating the daily amount ingested in a 0.5 per cent drug diet mixture died

TABLE 4

Results of in vivo testing with antimetabolites

Drug	Administration: Method	Avg. mg/Kg base daily	Period (days)*	No dead/total mice: Treated	No dead/total mice: Untreated	Avg. day of death: Treated	Avg. day of death: Untreated
2-4-diaminopyrimidines:							
pyrimethamine	0.5 mg. by gavage b.i.d.	42	0 to death	5/5	4/5	5.8	10.2
49–224	0.5 mg. by gavage b.i.d.	42	0 to death	5/5	4/5	6.6	10.2
48–210	0.5 mg. by gavage b.i.d.	42	0 to death	4/5	4/5	7.5	10.2
Parabens:							
hexyl p-hydroxybenzoate	0.5% in diet		—2 to 10	10/10	9/10	11.6	12.1
	1.5% in diet		—2 to 10	10/10	9/10	11.0	12.1
heptyl p-hydroxybenzoate	0.5% in diet		—2 to 10	10/10	9/10	11.4	12.1
	1.5% in diet		—2 to 10	10/10	9/10	9.4	12.1
Para-aminobenzoic acid	3% in diet		—3 to death	10/10	10/10	7.4	9.1
Sodium paraaminobenzoate	2% base in diet		0 to death	10/10	8/10	7.6	10.2
Phosphanilic acid	3% in diet		—3 to 18	6/10	10/10	10.6	9.1
	40 mg in water	2000	0 to 9	8/10	10/10	10.7	8.4
Metachloridine	7.5 mg b.i.d.	604	0 to death	10/10	9/10	3.8	9.0
Sulfadiazine	0.5% in diet		0 to 10	0/10	10/10	...	7.7

* Day 0 = day of inoculation.

earlier than the untreated mice. The fact that the drug was given in amounts approximating the maximum tolerated dose apparently contributed to the mortality of infected mice. Uninfected mice given the same amount of the drug were able to survive with slight weight loss.

Sulfadiazine, reported by Mayer, *et al.*[12] to be one of the more effective sulfa drugs against experimental histoplasmosis, also had a pronounced protective effect on our experimental infection (Table 4). Although all of the treated mice survived, 8 of the 10 survivors still harbored the organisms 4 weeks after the infection was initiated as shown by positive cultures of the liver and spleen.

The effect of similar sulfadiazine treatment of a sublethal infection at a time when the organism count was declining due to acquired immunity is seen in Table 5. Other results from our laboratory[2] and Rowley and Huber[15] have demonstrated that organism counts in mice with sublethal infections intravenously induced, decline after the second week of such an infection. It was expected that reinforcement of acquired immunity with treatment might eradicate the organism. However, the average organism count per gram of liver of treated mice was higher than that of untreated mice. Due to variation of individual counts, this difference between counts of the two groups was not significant. These results would support the hypothesis advanced by Mayer *et al.*[12] to explain the apparent lack of effect of sulfonamides on human cases. It is possible that the sulfonamides are particularly active in acute rapidly progressing infections such as the lethal infections given mice and that they are of less value in chronic infections where the multiplication rate of the organism has diminished.

TABLE 5

Results of treatment of a sublethal infection with sulfadiazine

Group	Number of mice	Administration			Organism count‡ avg. no. organisms per gram liver
		Method	Avg. mg/Kg. base daily	Period (days)*	
Infected; no treatment	10				1196
Infected; treated	10	0.5% in diet	590	14 through 23	2239

* Day 0 = day of inoculation.
‡ Killed for autopsy day 29.

Antibiotics: Nystatin had some protective activity when administered subcutaneously as a suspension in water as was shown earlier by Hazen and Brown[8] and Campbell *et al.*[3] When an attempt was made to put the drug into solution by using propylene glycol as a vehicle for parenteral administration, the toxicity was such that the treated mice died earlier than the controls (Table 6).

Amphotericin B was the most effective drug tested as judged by the amount of drug necessary for protection by both immediate and delayed treatment and by its sterilizing ability. Tests with amphotericin B in the form of an insoluble powder showed activity by both oral and parenteral routes of administration. Treatment could be delayed by half the expected survival time (4 days) and would still protect approximately 90 per cent of the mice from death. All cultures of spleen and liver were negative when a daily dose of 10 mg. per kilogram was given intravenously from day 4 through day 13 of the infection.

Eulicin, reported by other investigators to have an activity *in vitro*[19] but not *in vivo*[17] on *H. capsulatum* showed a protective effect on the experimental disease comparable to amphotericin B powder. When equal amounts of the two drugs were given intravenously, the delay in the average day of death was approximately the same for both treatment groups (Table 7). However, when an experiment was set up to compare the soluble desoxycholate form of amphotericin B with eulicin, the former was superior (Table 8). The soluble amphotericin B was completely protective after 5 doses of 1 mg. per kilogram, whereas eulicin at the same dosage decreased the mortality only slightly. At the same time, therapy with eulicin of mice infected with a second strain of *H. capsulatum* (the M strain) showed no protective effect (Table 8). This difference in strain susceptibility may explain the lack of *in vivo* effectiveness reported by Solotorovsky *et al.*[17] A further experiment was set up to compare the activity of eulicin intramuscularly administered with the same amount of eulicin and amphotericin B desoxycholate given intravenously. Table 9 shows that eulicin was of the same order of activity when given by either the intramuscular or intravenous route. Most of the mice treated with eulicin by either route were protected from death, but all survivors had organisms in their liver and spleen cultures. The negative results of liver and spleen cultures in the

TABLE 6

Results of treatment with nystatin

Drug administration route	Form of drug	Period (days)*	Avg. mg/Kg. daily	No. dead/total mice Treated	No. dead/total mice Untreated	Avg. day of death Treated	Avg. day of death Untreated
Subcutaneous	1 mg in water b.i.d.	0 to 3	88.8	4/10	8/10	23.5	10.2
Intraperitoneal	0.2 mg in propylene glycol	0 to 2	9.0	10/10	10/10	5.9	7.8
	0.2 mg in propylene glycol	4 and 5	11.0	10/10	10/10	7.4	7.8
Intravenously	0.2 mg in propylene glycol	4, 5, 7	10.9	11/11	10/10	7.3	7.8

* Day of inoculation = 0.

TABLE 7

Comparison of eulicin sulfate and amphotericin B given intravenously

Drug group	Daily dose per kilo	Treatment period‡	No. dead/total mice Treated	Untreated	Average day* of death Treated	Untreated
Eulicin sulfate†	1 mg.	day 0 to day 9	8/10	10/10	13.0	6.5
Amphotericin B‡	1 mg.	day 0 to day 9	10/10	10/10	13.5	6.5

* Day of inoculation = 0.
† Made up in physiological saline and refrigerated. Approximately 90% base.
‡ Made up in 5% dextrose every other day. 1 mg. = 1000 activity units.

TABLE 8

Comparison of eulicin sulfate and amphotericin B desoxycholate given intravenously

Drug group	Daily dose per kilo	Days* of treatment	No. dead/total mice Treated	Untreated	Average day* of death Treated	Untreated
Standard strain:						
Amphotericin B	4 mg.	1, 3, 5, 7, 9	0/8	6/6	...	8.8
desoxycholate†	2 mg.	1, 3, 5, 7, 9	0/10	6/6	...	8.8
	1 mg.	1, 3, 5, 7, 9	0/9	6/6	...	8.8
Eulicin sulfate‡	4 mg.	1, 3, 5, 7, 9	4/8	6/6	19	8.8
	2 mg.	1, 3, 5, 7, 9	8/10	6/6	12	8.8
	1 mg.	1, 3, 5, 7, 9	9/10	6/6	10.2	8.8
M strain:						
Eulicin sulfate‡	2 mg.	1, 3, 5, 7, 9	9/9	6/6	8.7	8.0

* Day of inoculation = 0.
† Made up in 5% dextrose just before use each day.
‡ Made up in 5% dextrose before experiment and refrigerated. Approximately 90% base.

TABLE 9

Comparison of treatment by eulicin given intravenously and intramuscularly with amphotericin B desoxycholate given intravenously

Drug group	Administration: Method	Daily dose per kilo	Period days*	No. dead/ total mice	Culture results: no. positive/total
Eulicin sulfate‡	IM	2 mg.	0–4, 7–11	0/10	10/10
Eulicin sulfate‡	IV	2 mg.	0–4, 7–11	1/8	7/7
Amphotericin B desoxycholate‡	IV	2 mg.	0–4, 7–11	0/7	0/7
None, controls	..	...	.....	10/10	...

* Day of inoculation = 0.
† Killed for autopsy 4 weeks after inoculation.
‡ Amphotericin B made up every other day in 5% dextrose, held in refrigerator for 24 hours to decrease toxicity. Eulicin made up once before the experiment and refrigerated.

group of mice treated with Amphotericin B desoxycholate again indicated its superiority to Eulicin.

After the reports[6,14] of the synergism between human gamma globulin and antibiotics in the treatment of experimental bacterial infections, we attempted a similar combination of therapy against *Histoplasma* infections. In order to obtain immune globulin which would be likely to contain antibodies against histoplasmosis, we used that produced commercially by a midwest firm which uses placental extracts as a source of the globulin, each lot representing material from 20,000 to 30,000 individuals. It is true that circulating antibodies would be expected to have less effect on organisms such as *H. capsulatum* within cells of the reticulo-endothelial system, than on bacterial septicemias, but it is conceivable that the spread of the disease could be affected by such antibodies. However, inspection of Table 10 shows that human immune globulin had no effect in addition to that exerted by amphotericin alone. Three different dosage levels of amphotericin B were combined with immune globulin (Groups 1b, 2b. and 3b, Table 10) and in every case, the mortality of infected

TABLE 10

Effect of combined treatment using human gamma globulin and amphotericin B

Group no.	Treatment	Administration: Method	Daily dose per kilo	Period (days)*	No. dead/ total mice	Average day of death*
1a.	Amphotericin B†	IV	4 mg.	0 to 9	0/10	...
b.	Amphotericin B + gamma globulin‡	IV IP	4 mg. 10 ml.	0 to 9 day 0 only	0/8	...
2a.	Amphotericin B	IV	1 mg.	0 to 9	10/10	13.5
b.	Amphotericin B + gamma globulin	IV IP	1 mg. 10 ml.	0 to 9 day 0 only	10/10	11.7
3a.	Amphotericin B	IV	0.25 mg.	0 to 9	10/10	7.4
b.	Amphotericin B + gamma globulin	IV IP	0.25 mg. 10 ml.	0 to 9 day 0 only	10/10	7.6
4.	Gamma globulin	IP	10 ml.	day 0 only	10/10	6.5
5.	None, controls	..	....	.....	10/10	6.5
6.	Inoculum incubated with gamma globulin	IV	7.5 ml.	day 0 only	5/5	8.8
7.	Inoculum incubated with physiological saline + gamma globulin	IV IV	.... 7.5 ml.	..... day 0 only	... 5/5	... 6.8

* Day of inoculation = 0.
† Amphotericin B powder made up in 5% dextrose every other day, refrigerated. 1 mg. = 1000 activity units.
‡ Gamma globulin was human immune globulin lot no. 172212, Pittman-Moore Company.

mice was similar to that obtained by comparable doses of the drug alone (Groups 1a, 2a, and 3a, Table 10). Gamma globulin treatment alone (Group 4, Table 10) resulted in the same degree of mortality as was experienced by untreated control mice (Group 5, Table 10). The only beneficial effect was obtained by incubation of the organisms for 30 minutes at 37° C in the immune globulin before inoculation (Group 6, Table 10). Controls for this procedure (Group 7, Table 10), were inoculated with organisms incubated for 30 minutes at 37° C in physiological saline. Immediately afterwards, they were inoculated intravenously with an amount of gamma globulin comparable to that present in the mixture of gamma globulin and organisms given to Group 6. A more pronounced neutralization of the infecting organisms was obtained when the experiment was repeated (Table 11). Incubation of the organisms with immune globulin before inoculation prevented death of any of the inoculated animals although all were found to be infected at autopsy 4 weeks later.

Summary

A number of drugs including quinolines, acridines, antimetabolites, antibiotics and miscellaneous drugs were tested against a standardized infection produced with intravenously injected yeast phase of *Histoplasma capsulatum* in white mice.

Ethyl vanillate, formerly used for treating human infections was not therapeutically effective in the experimental infection.

Nystatin, eulicin, and amphotericin B, of the antibiotics, had protective effect. Amphotericin B in its soluble desoxycholate

TABLE 11

Neutralization of a lethal dose of H. capsulatum *by human immune globulin*

Treatment of inoculum	Administration of gamma globulin*	No. dead/ total mice	Average day of death
Inoculum incubated with gamma globulin	with inoculum	0/10	..
Inoculum incubated with physiological saline	immediately after inoculum	10/10	9.7
No incubation	6 hours after inoculum	8/8	8.8
No incubation	no gamma globulin	10/10	10.0

* Gamma globulin (human immune globulin, Lot 172212, Pittman-Moore Company) was given intravenously in the amount of 7.5 ml./K.

form was the most effective of any drug tested and was capable of sterilizing a lethal infection. The soluble form of amphotericin B was superior to eulicin when equal amounts were administered intravenously. Amphotericin B as the insoluble powder, on the other hand, had a degree of activity approximately equivalent to eulicin.

Of the antimetabolites tested, phosphanilic acid and sulfadiazine had some protective effect. Both of these drugs are known to be para-aminobenzoic acid antagonists. Sulfadiazine was completely protective although it did not sterilize a lethal infection. However, sulfadiazine did not decrease the numbers of organisms recoverable after treatment of a declining sublethal infection.

Human gamma globulin did not potentiate the activity of amphotericin B against the experimental infection. Neutralization of a lethal infecting dose could be demonstrated when the organisms were incubated with human gamma globulin before inoculation.

Acknowledgments

The authors are indebted to E. R. Squibb and Sons for the ethyl vanillate, nystatin and amphotericin B powder; to Merck, Sharp and Dohme for eulicin; to Heyden Newport Chemical Corporation for the hexyl and heptyl esters of p-hydroxybenzoic acid; to Eli Lilly and Company for the phosphanilic acid; to Burroughs Wellcome and Company for the 2–4 diaminopyrimidines and to Winthrop Stearns and Company for the primaquine used in these studies.

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MESOTHELIOSARCOMA OF THE PERICARDIUM

C. DE CHENAR[a]

The literature of mesothelial neoplasia represents one of the controversial chapters of tumor pathology, with interpretations of findings from diametrically opposite angles: from the confirmation of accepted facts to the denial of any such tumor.

Even those who accept the reality of the tumor differ in its classification, therefore, there is no unanimous agreement. Stout and Himadi (35) collected in their review a series of terms used by investigators describing the microscopic appearance of serosal tumors as follows: fibroma (16), fibrosarcoma (14), fibrosarcoma myxomatodes (29), myxosarcoma (26), leiomyosarcoma (6), sarcoma (21), sarcoma-like tumor (25), endothelioma (23) and endothelial sarcoma (24). Further terms used are: coelothelioma (1) and mesothelioma (30). Most of these terms, indicate the presumable histogenesis of the tumor. The microscopic structure and the invasiveness of the tumor to be described here, might be more accurately reflected by the term "mesotheliosarcoma."

More on the negative side is the opinion of Willis (38) that "... while the possibility of occurring neoplasms cannot be denied, the great majority of cases so reported are certainly only examples of serosal disease secondary to undiscovered primary tumors in neighbouring viscera."

The rigid refusal comes from Fischer-Wasels (17) in this sentence: "The existence of tumors of serosal endothelium has never been established and such tumors present all the appearances of carcinoma." He simply ignores the results of tissue culture experiments of Maximow (28), published five years prior to his paper. More than two decades later, Sano and co-workers (31) brought further evidence to the histogenesis of mesothelioma.

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Embryological Background

The participation of the three mesothelial membranes in tumor formation is not alike. While more than a hundred pleural tumors had been reported, fewer peritoneal growths were found and, only about a dozen cases are known where the pericardium was the substratum of origin of a mesothelial blastoma (4, 5, 7, 8, 10, 11, 12, 13, 15, 20, 30, 32, 33, 37). These cases show the predominance of epitheloid cells and thus indicate the primary participation of the mesothelial lining of the pericardium, leaving the fibrous elements of the underlying layer in the background.

Most of the authors who accept coelomic tumor formation as an autogenous process, presume that embryonal aberrations of the serosal meothelium are the responsible oncogenic factors.

The phylo- and ontogenetic development of the pericardium is so complex that a brief review of the process is justified for the clarification of the possible mechanism of tumor formation. Developmental disorders, in general, have their location of preference at areas where either a coalescence of different germ layers or a fusion of tissue primordia occurs. The latter is the case in the differentiation of the pericardium. This process, as summarized by Arey (2), Hertwig (22) and Staemmler (34), is initiated by a folding of the splanchnopleura which covers the omphaloenteric veins, shortly before the fusion of the cardiac vesicles. The folds tend to establish temporary mesocardia. The cardiac fusion extends both caudally and in the lateral direction, therefore, the "cavité pericardiaque primitive" of Brachet has a transverse foramen dorsally and communicates with the rest of the coelomic cavity on both sides. In the next step, a mesodermal constriction, as pleuropericardiac membrane, surrounds the ductus Cuvieri, as soon as the umbilical veins prepare their way into the sinus venosus. This latter development results in the ventral attachment of the pericardium to the parietal structures in the form of the mesocardium anterius. Upon formation of the myoepicardial mantle, the mesocardium anterius undergoes rapid regression. The most heterogenous part of the pericardium is the ventral portion where an open gap remains at the time when the septum transversum develops and where, in the majority of known cases of primary pericardial tumors, the initial development of the growths took place.

The ontogenetic development might well predispose the antero-rostral pericardium to neoplastic proliferation, but another factor, the embryonal pluripotentiality of the mesothelium, cannot be disregarded in a study of the formal genesis of possible tumors.

Maximow (28) found that explanted normal mesothelial cells could assume the shape and the fiber forming activity of fibroblasts. He also concluded that mesothelial lining cells may develop into epithelial and connective tissue cells. Sano *et al.* (31) arrived to a similar deduction while observing tissue cultures. They write that ". . . mesothelial cells may grow in membranes, or they may assume a more epithelial-like form, as the result of irritation." Maximow (28) also confirmed this finding by stating: "In inflammation, they become elongated and spindled like fibroblasts." Stout and Murray (36), in a descriptive and experimental study (tissue culture), conclude that the lining cells of the pleura represent the primary source of mesothelial tumors of this serous membrane.

The microscopic appearance of mesothelial tumors gives a multiform variety of mesenchymal and epithelial patterns, between the extremes of sarcoma and carcinoma, with widely different participation of stromatic components. So far, three characteristic types have been established, namely, (a) epithelial, (b) fibrous and (c) mixed tumors. The case to be presented here belongs to the first group.

Case Report

Case No. 26461, a 73 year old white woman. She was a patient in the hospital for more than four years. Her psychiatric diagnosis was manic depressive psychosis. At the time of admission, as well as at annual routine examinations, the roentgenologic, laboratory and serological studies gave negative results. Her first complaints of a vague, retrosternal, pain began one year prior to death. At that time, all the above examinations failed to demonstrate any pathological change. Repeated examinations during the next six months remained unsuccessful, until one half year before death, a skiagram disclosed an "increased cardiac axis". A hemogram included 7% myelocytes and metamyelocytes; hemoglobin was 10 gm. Sedimentation rate by the Westergren's method was 6 mm in the first hour. Nonprotein nitrogen was 52 mg per 100 ml. This last finding was in line with a rapidly progressing renal insufficiency on an arteriosclerotic basis. After five more weeks, the roentgenologist found that the density of the right hilus was increasing and, the appearance was suspicious of a lung tumor. From this time on, the pain radiated toward the epigastrium. There was a change in the hemogram,

including a slight decrease in the number of erythrocytes and an increase of the leukocytes with 15% juvenile forms. The amount of hemoglobin was unchanged; the sedimentation rate doubled. Nonprotein nitrogen increased to 144 mg per 100 ml. During the last two months of life, the cardiac enlargement was evident by physical examination. There was, however, no sign of obstruction of the major blood vessels about the heart. There was no dyspnea until a few weeks prior to death, when a severe uremia deteriorated the physical condition of the patient. She needed tube feeding and intravenous infusions. She had more and more sereve, rather diffuse, thoracic pain. Her mental depression became aggravated by a constant fear of impending death. The fatal exitus was precipitated by uremia, due to advanced nephrosclerosis.

Anatomical Findings

The anterior mediastinal structures were fused ventrally with the sternal periosteum and costal perichondrium, as well as with the perihilar connective tissue; and posteriorly, with the parietal pericardium and epicardium of the right atrium and ventricle. This growth was dense, elastic, pale yellowish-gray, with a few small areas of necrosis. The heart was deformed by it to a somewhat spherical shape, and its weight was 510 gm. There was no continuous pericardial cavity left on the sternal surface, just pouches of various dimensions, as shown on Fig. 1, in the left upper quadrant. The rest of the pericardial space contained 140 ml of a turbid, sanguinolent, fluid with slowly sedimenting floccula of tumor particles. Both layers of the serosa showed a diffuse, laminar, spreading of the tumor, accompanied by an exudative-adhesive inflammation. There was no involvement of either pleural cavity, although an area of about 5x8 cm of the median aspect of the middle lobe of the right lung was adherent to the tumor. It was indented, without being infiltrated, by several sharply outlined, hard, nodular protuberances of the tumor (Fig. 1). On the surface of the neoplasm the anatomical, as well as the later histological, examination failed to demonstrate any invasion of the surrounding tissues by its contiguous parts, as the surface was hard and fibrous. To the contrary, there was a deep penetration into the myocardium, through the considerably thickened wall of the right atrium and ventricle, into the adjacent interventricular septum, with a rather clear borderline between the tumor and the myocardium. The atrioventricular girdle was also heavily invaded. The major blood vessels were not compressed, but their walls were hardened by the tumorous infiltration. On

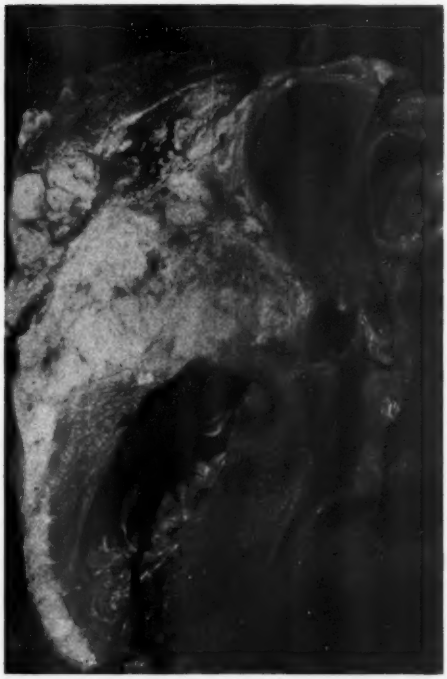

FIG. 1. Section of the pericardial tumor through its longest diameter, penetrating the right atrium, part of the right ventricle and surrounding the large blood vessels. Sharply limited protrusion toward the lung, without infiltration.

incision, several small areas of necrosis discharged a thick, yellowish-red fluid.

A careful anatomical, and later histological, examination of the lung, gastrointestinal system, meninges, endocrine and hemopoietic apparatus and the rest of the viscera remained unsuccessful in finding another, eventual primary or secondary, tumor. Non-contributory pathological changes in other organs included generalized arteriosclerosis, diffuse cerebral atrophy (1050 gm), severe arterial nephrosclerosis and chronic cystitis.

Histological Findings

The relation between the tumor and non-neoplastic stroma is variable. Large sectors of the surface are encapsulated by a dense,

fibrous, tissue, into which tumor cells do not penetrate in a considerable number to any distance, but which shows areas of infiltration by small round and plasma cells. At many points, trabecular extensions of this inflammatory connective tissue might be followed toward deeper parts of the tumor, where they undergo hyalinous degeneration and gradual necrosis.

The participation of blood vessels in the stroma is different in areas of active proliferation from that found in fields of slow growth or regressive changes. In the former case, irregular, defective, vascular cavities developed, with only traces of an incomplete endothelium, but more frequently, without any such lining. In these pseudovascular spaces, the tumor cells are freely exposed toward the lumen. Some of these spaces contain aggregates of proliferative fragments (Figs. 2, 3 and 4). In the zones of moderate activity, the spaces which contain blood are less numerous but they have a rather continuous endothelial lining and an extensive perivascular stroma. No channels were found which could be regarded as lymphatic capillaries, only some intercellular spaces, without an endothelium, between smaller cell groups (Fig. 5). There are only very few fibroblasts in the areas of proliferation, while in the less active fields, a framework of dense connective tissue is frequent.

The tumor cells represent two types of differentiation: (a) in the areas of extension, those of polygonal, rather epitheloid appearance, with acidophilic cytoplasm and mostly large, hypo- or hyperchromatic nucleus. The nuclei display various patterns of asymmetric or multipolar division. Mesothelial tumors with these characteristics were often described as endothelial sarcoma, without proper reference to the mesothelial membrane. (b) In the less active zones, a small number of cells is similar to fibroblasts. They are elongated, fusiform and frequently aggregated

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FIG. 2. Moderately vascular portion of the tumor from th posterior aspect of the right atrium. Chronic inflammatory reaction on the surface. Van Gieson's method, 120x.

FIG. 3. Rich network of irregular, incomplete, vascular channels from the tumor within the interventricular septum. Ehrlich's hematoxylin, 120x.

FIG. 4. Pseudovascular channel from near the surface of the right ventricle, with concentric orientation of the tumor cells. Several karyokinetic formations. Spielmeyer's hematoxylin, 470x.

FIG. 5. Communicating pseudovascular channels from the right atrial portion of the tumor. Ehrlich's hematoxylin, 440x.

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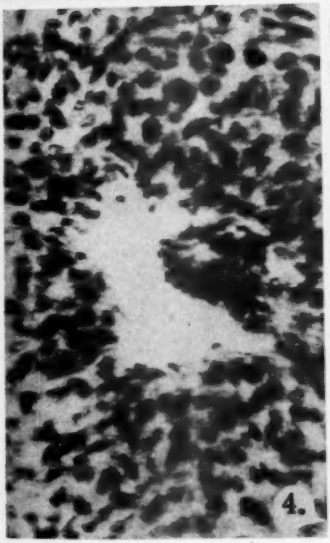
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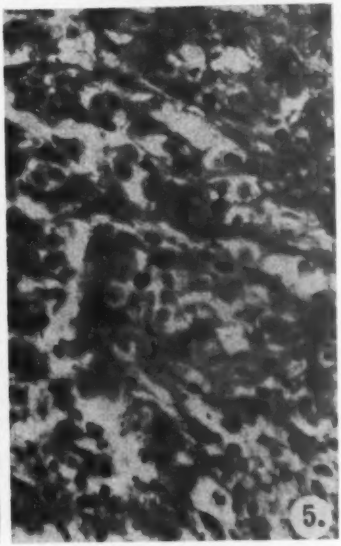
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in groups around perivascular spaces where they are surrounded by collagenous fibers (Fig. 5). Tumors composed of this type of cells were mentioned as fibrosarcoma. In the present case, their participation in the structure of the tumor is rather exceptional.

In regressive parts of the tumor, adipose and tyalinous degeneration affects the cells, before those disintegrate, as the result of central necrosis.

On the epicardial surface of the tumor, there is a heavy proliferation of the serous membrane, with a gradual transition into the tumor itself (Fig. 2).

Along the zone of contact of the tumor with the myocardium, in some areas, there is no defensive reaction in the latter (Fig. 6), while in most fields, there is a definite inflammatory infiltration in the myocardium.

In wide areas of the contacting myocardium, nuclear and cytoplasmic changes occur, following the pattern of the phenomenon of Gurwitsch. (3, 19)

As mentioned previously, the neighboring organs and the regions of routine metastases did not contain any tumor, neither as an eventual primary source to the neoplasm of the heart, nor as a secondary growth. In a mediastinal lymph node, however, about 2 cm above the rostral pole of the tumor, in a perihilar lymph node, several small groups of cells of epitheloid appearance were found. These were similar to the cells of the proliferating parts of the cardiac tumor (Fig. 7).

The mesothelial character of the tumor is evident in sections prepared with Gömöri's silver impregnation method, showing an argentophilic reticular network in most fields without necrosis (Fig. 8).

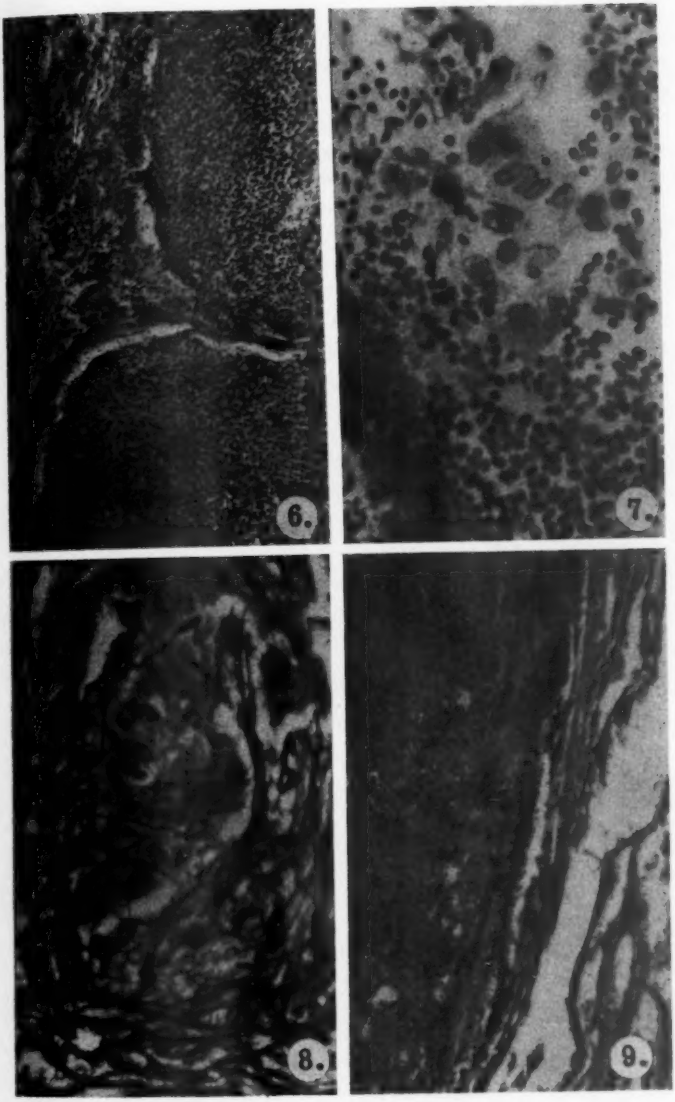
In the deep portions of the tumor, there are areas of necrosis, in various dimensions. The smaller ones, a few hundred microns in dimensions, are in the center of massive conglomerates of cells.

Fig. 6. Contact of tumor with ventricular myocardium. No significant defensive reaction. Ehrlich's hematoxylin, 120x.

Fig. 7. Mediastinal lymph node. Infiltration by a small group of tumor cells. Mallory's hematoxylin, 430x.

Fig. 8. Argentophilic intercellular reticulum, from near the surface of the tumor. Gömöri's silver impregnation, 380x.

Fig. 9. Contact of the tumor with the lung. Compression and partial atelectasis of the lung; subpleural anthracosis. Ehrlich's hemotoxylin, 120x.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Nearly the entire tumor is rather compact, except in those parts which deeply penetrate into the myocardium. These infiltrating cells, in a more or less loose arrangement, are of epitheloid character (Fig. 5).

There is a clear distinction along the outlines of the whole growth toward the surrounding tissues. Even at those points where the lung is indented by the tumor, a separation of the two different structures can be seen, with some compression or atelectasis of the lung (Fig. 9).

Comment

The above described tumor is composed of cells of epitheloid character arranged in irregular masses, forming pseudovascular channels and supported by an argentophilic intercellular reticulum. On its entire surface, it invades the myocardium. The mutual reaction of the two tissues is far from a uniform pattern. In some areas, they are side-by-side, without any visible change, but in the majority of fields, there is an acute myocarditis with mixed infiltration and advanced myodegeneration. In juxtaneoplastic zones, the phenomenon of Gurwitsch is significant: the cytoplasm of the myocardium turns toward a definite basophilic reaction and the nuclei enlarge up to five times in diameter of that of unaffected fibers. The chromatin substance of these megalonuclei is disorganized, taking the form of irregular granules or filamentous precipitates. Gurwitsch attributed these parenchymal alterations in tissues contacting certain malingnant tumors to a "mitogenetic radiation" of the neoplasm. Such tumors, in tissue cultures, might greatly speed up schizomycetic fission. It is not the task of this study to elaborate on the pathophysiological implications of this phenomenon, whether it is a primary proteoglycolytic enzymatic effect, or in a way related to the principle of photoluminescence, but merely to mention the distant effect of this tumor upon the myocardium.

The genuine mesothelial tumors have been known as nonmetastazing ones. Any trace of a metastasis is considered as a rarity. In the present case, there was no metastasis to any organ, just an insignificant spreading to a regional lymph node.

As a conclusion, the subject of the above study can be classified as a mesothelial neoplasia of the pericardium. The present knowledge, supported by experimental evidence of explantations, offers

sufficient confirmation that lining cells of serosal cavities are able to develop into those with morphological similarities to epitheloid cells or fibroblasts. However, the fundamental question, whether these derivatives, if they occur in tumors, are the result of an oncogenous or of a dysontogenetic "modus generandi", remains open. In a similar way, authors of cases so far reported cannot always arrive to the decisive differentiation, whether such tumors should be regarded as carcinomatoid or sarcomatoid growths. In the case of this study, as outlined above, there is evidence of sarcomatous structure. It might be assumed that in mesothelial neoplasia, the lining cells of the coelomic epithelium supply the primary substratum for the anaplastic dedifferentiation, although an eventual participation of the endothelium of lymphatic vessels had not been definitely excluded in every case. At any rate, for tumors of the antero-rostral pericardium, an ontogenetic aberration of this embryologically complex structure is the most plausible explanation, as far as the theory of Cohnheim holds that endogenous factors, as a result of mutation, motivate neoplastic formations.

Summary

A case of heterotypic mesothelioma of pericardial origin is presented. Its extension over the serous membrane and its invasion of the myocardium are similar to those of the "cancer en cuirasse." A careful search for an eventual primary or secondary tumor in other organs remained unsuccessful. After an embryological background, an anatomical and histological description is given in detail. A critical and comparative evaluation of the findings is attempted, with a conclusion as to the possible origin and formal genesis of the neoplasm. The term "mesotheliosarcoma" is introduced for the histogenetic designation of the tumor.

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EFFECT OF HEPARIN ON NEOMYCIN[a]

R. D. HIGGINBOTHAM[b]

Heparin is a strongly acidic macromolecule which is stored in relatively high concentrations in the characteristic granules of the tissue mast cells (9). This sulfated mucopolysaccharide is released from these cells into the connective tissues in reponse to various types of local injury (12). The significance of this cellular reaction has been the subject of a great deal of speculation. It is of interest that heparin has been reported to interact with a variety of substances having physiologic or pharmacologic importance (14, 1) and, in some instances, to neutralize the toxicity of the very substances which are responsible for initiating the release of the heparin from the mast cells (8). Since this biologically active substance is released within or in proximity to the area of injury, it has been suggested that heparin may be an important factor in mechanisms of local resistance. It has been of interest in this respect that heparin-treated mice have an enhanced resistance to otherwise lethal doses of certain toxic metabolites of microorganisms such as, polymyxin B, neomycin, viomycin and dihydrostreptomycin (8).

Previous studies with polymyxin B and other toxic drugs have suggested that the protective effect of heparin is related to its ability to form acid-base complexes with these toxic cationic agents *in vivo* and in this manner reduce the active concentration of these noxious agents in the tissues. This idea has received support from the recent studies of Mora, Young and Shear (10) who have recently reported that sulfated derivatives of a synthetic polymerized glucose preparation will also protect mice against certain of these toxic cationic agents. These investigators noted that the effectiveness of these acidic polymers was dependent on their charge density.

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The present study has dealt with the interaction of heparin with neomycin and several other toxic antibiotic agents both *in vitro* and *in vivo*. Utilizing both toxic and antibiotic properties of neomycin as indicators of heparin binding, it was found that heparin had a differential effect on these two properties of the drug. Heparin neutralized the acute toxic effects of neomycin but this loss in toxicity was not accompanied by an equivalent loss in antibiotic activity and heparin was found to enhance the prophylactic value of neomycin in mice infected with *Staphylococcus aureus*.

Materials and Methods

CBA mice, mixed sexes, 2 to 3 months of age and ranging in weight from 19 to 21 grams in weight were used throughout this study. Drugs employed were: Na heparinate (117 μ/mg, Upjohn Co.[a]) and the sulfate salts of polymyxin B (Aerosporin, Burroughs Wellcome & Co.[a]) viomycin (Vinactane, Ciba), neomycin and dihydrostreptomycin (Nutritional Biochemical Corp'n.). Drugs were prepared in deionized water for *in vitro* studies and in isotonic, pyrogen-free, sterile saline for *in vivo* studies. All drugs used for injection were administered by the intravenous route (caudal vein), either singly or in combination, in a volume of 0.25 ml.

Bacteria: A previously described mouse-virulent strain of *Staphylococcus aureus* (5), originally isolated from a lesion in a human, was used to measure persistence of the antibiotic activity of neomycin in mice. The bacterial cells were prepared for injection by growing them for 18 hours on a nutrient agar slant and then transferring this growth to brain-heart infusion broth for an additional 3 hours growth. The cells were harvested from this media by centrifugation then washed once and resuspended with sterile 0.85% saline. A Klett-Summerson photocolorimeter with a 420 mμ blue filter was used to adjust the concentration of the bacterial suspension to a predetermined turbidity equivalent to approximately 6 x 10^8 viable cells/ml. Twenty-five hundredths ml of this concentration of bacteria was uniformly fatal to mice when injected by the intraperitoneal route and was used as a standard challenge dose.

Results

Dye release studies. The relative affinity of heparin for the various cationic antibiotic agents was determined by means of a previously published technique (6) employing the cationic dye, toluidine blue, as an indicator. This dye exhibits the property of metachromasia, having a shift in its optimal absorption from 630 mμ in the "free" state to 540 mμ when bound to heparin. Displacement of this cation from the acidic groups on heparin by other cationic substances yields the "free" dye whose concentration can then be determined colorimetrically in the

presence of the bound dye. The interaction of these substances is an ion exchange reaction in which heparin serves as the ion exchanger and the test drug can be described as a "releaser" agent.

In Figure 1, the amounts of dye released for each of the graded amounts of the various antibiotics have been plotted against the amounts of the respective antibiotic employed. It can be seen that they exhibited varying degrees of releaser activity. For comparative purposes, the amount of drug required to release 50% of the bound dye has been used to describe the relative affinities of heparin for the various cationic agents; *i.e.*, the affinity is inversely related to the amount of cation required. The values for neomycin, polymyxin B, viomycin and dihydrostreptomycin were: 20 μg, 30 μg, 630 μg and 4000 μg (estimated), respectively. Although it is difficult to relate these quantitative differences to any biological property of these drugs, it is considered significant that a characteristic mucopolysaccharide of the connective tissue has the potential to form complexes with these noxious agents. The possibility thus exists that heparin or other acidic

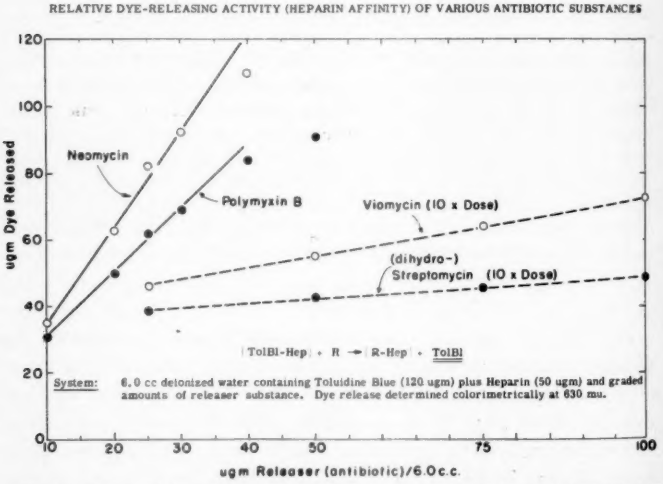

FIG. 1. Comparison of the dye-releasing activities of graded amounts of cationic antibiotic agents. Note that all substances complex with heparin as shown by their release of toluidine blue and that amounts required for activities by viomycin and dihydrostreptomycin are 10 times the amounts indicated on the abscissa.

mucopolysaccharides can form complexes with such noxious agents diffusing through the connective tissue between blood and parenchymal tissue.

Toxicity studies. In previous work, the *in vivo* stability of complexes of heparin with polymyxin B and other noxious cationic drugs has been suggested by the decreased toxicity of a given dose of a noxious agent when injected intravenously in mixture with suitable amounts of heparin (4). Since intravenous challenge with such noxious agents is a dose response effect and results in either a very rapid death or rapid recovery (*i.e.*, usually within 30 minutes), survival of the test animals for 24 hours after challenge has been considered as an adequate criterion for demonstrating neutralization of the toxic agent. A similar study with neomycin-heparin complexes has been performed. The dose of neomycin used was 40 μg/gm and was equivalent to approximately 1.6 $LD_{50's}$. The dose response range for neomycin was found to lie between 15 and 30 μg/gm and the dose of neomycin used in this study was therefore in excess of a minimal lethal dose. Accumulated results of two experiments are shown in Table 1. In these experiments the degree of neutralization achieved by heparin against the challenge dose of neomycin was directly related to the amount of heparin employed. Comparison of these results with the neomycin dose-response curve suggested that approximately 1 μg of heparin neutralized the toxicity of 1 μg of neomycin.

For comparative purposes, results obtained with neomycin and representative results obtained in similar studies with other toxic antibiotics have been presented in Table 2. It is obvious

TABLE 1

*Effect of Heparin on toxicity of Neomycin in Mice**

Dose of Heparin	Survival ratio	Per cent survival
(μg/gm)	(S/T)	
control	0/10	0
5	0/20	0
10	4/20	20
15	8/20	40
20	16/20	80
25	20/20	100

* Mice were challenged (i.v.) with a single lethal dose of neomycin (40 μg/gm) in mixture with one of the graded amounts of heparin and contained in 0.25 ml saline.

TABLE 2

*Effect of heparin on toxicity of various cationic antibiotics in mice**

	Challenge dose		Survival ratio
1. a.	Polymyxin control	5.0 μg/gm	0/10
b.	Polymyxin heparinate	5.0 μg/gm 2.5 μg/gm	10/10
2. a.	Neomycin control	40 μg/gm	0/15
b.	Neomycin heparinate	40 μg/gm 25 μg/gm	15/15
3. a.	Viomycin control	200 μg/gm	0/10
b.	Viomycin heparinate	200 μg/gm 25 μg/gm	9/10
4. a.	Dihydrostreptomycin control	225 μg/gm	1/10
b.	Dihydrostreptomycin heparinate	225 μg/gm 75 μg/gm	8/10

* Heparin and drug administered as a single (i.v.) injection of the complex contained in 0.25 ml saline.

that the neutralization of neomycin toxicity by heparin is not a unique phenomenon and can be demonstrated with other antibiotics that form complexes with heparin. In each instance, heparin neutralized a significant amount of the toxicity of these agents. From the observations made in these and other studies (8), it appears that the neutralizing effect of a given amount of heparin varies with the dose and nature of the particular toxic agent employed and that heparin is more effective when mixed with the toxic agent prior to injection than when administered separately. The ability of heparin to neutralize the toxicity of these diverse toxic metabolites of microorganisms would support the view that this tissue mucopolysaccharide may play an important role in natural resistance of tissue to injurious stimuli.

Antibiotic studies. The influence of heparin on the *in vivo* activity of neomycin was further investigated by determining its effect on the antibiotic activity of this drug following a single intravenous administration of neomycin with and without heparin. A large number of mice were divided into three groups and received one of the following treatments: 1) 20 μg/gm neomycin, 2) 20 μg/gm neomycin plus 25 μg/gm heparin or 3) 40

μg/gm neomycin plus 25 μg/gm heparin. Individual groups of 10 animals in each were given one of the neomycin preparations at 5, 4, 3, 2, 1, or zero hours prior to intraperitoneal challenge with 0.25 ml of saline containing a lethal dose of a mouse virulent strain of *Staphylococcus aureus*. This organism characteristically produced a fatal bacteremic infection within 6 to 24 hours after intraperitoneal challenge (5). Experiments could therefore be terminated at the 24 hour period and the protective effect of neomycin determined.

The 20 μg/gm neomycin dose was immediately lethal to approximately 10% of the animals receiving this amount without heparin and this dose was therefore in excess of the maximal amount tolerated as a single treatment by the intravenous route. In contrast, no evidence of lethal antibiotic toxicity was noted in either of the heparin-treated groups, indicating that 25 μg/gm heparin neutralized the lethal effects of a minimum of 20 μg/gm neomycin (B, C). However, it can be seen in Table 3 (A vs. B) that heparin did not markedly impair the measurable antibiotic activity of this dose of neomycin in the 0–2 hour period and produced only a modest reduction in antibiotic effect in the 3 and 4 hour periods. The data represents an accumulation of results from three experiments and in each instance a similar degree of inhibitory effect was noted. This would suggest that the neutralization of a given amount of neomycin by heparin is not necessarily accompanied by a neutralization of an equivalent amount of antibiotic activity. This was supported by the observa-

TABLE 3

Effect of heparin on antibiotic activity of neomycin in mice infected with Staphylococcus aureus

Treatment groups	Time of treatment prior to infection (hours): 0–2	3	4	5
	(Survival ratios)			
A. Neomycin-Saline 20 μg/gm	30/30 (100%)	15/27 (56%)	6/27 (22%)	0/17 (0%)
B. Neomycin-Heparin 20 μg/gm.25 μg/gm	30/30 (100%)	6/17 (35%)	3/27 (11%)	0/17 (0%)
C. Neomycin-Heparin 40 μg/gm.25 μg/gm	30/30 (100%)	25/26 (96%)	22/27 (82%)	11/17 (65%)

* Treatments administered as single i.v. injection (0.25 ml saline) at noted intervals prior to i.p. challenge with lethal dose of *S. aureus*.

tions that 40 μg/gm neomycin (C), of which a minimum of 20 μg/gm had been neutralized by heparin, was more active than 20 μg/gm neomycin with or without heparin. This effect was most pronounced at the 5 hour period where 65% of the animals receiving the maximal tolerated dose of neomycin-heparinate were protected in contrast to no protection at this period in animals given the maximal tolerated dose of neomycin without heparin (C vs. A).

It is thus quite apparent that heparin did not impair the antibiotic effectiveness of neomycin to the same extent that it reduced the acute toxic effects of this drug in the animal. Because of this, relatively larger doses of neomycin were tolerated with a concomitant increase in measurable antibiotic activity and prolongation of effect of a single dose.

Discussion

The present experiments may be considered as model systems demonstrating three associated potential activities of heparin in the tissues: 1) ion exchange, 2) neutralization of toxic substances and 3) release of a biologically active agent. Each of these activities would represent different aspects of a single function of heparin in which it serves as a molecular site for both binding and releasing of cationic agents. As described elsewhere (5), the efficiency of heparin as an ion exchanger would be governed by the relative concentrations of the cations concerned as well as the dissociation constants of their respective heparin complexes. The influence of such interactions on various biologic phenomena is implicit in the recent studies of Mora and Young (11) who have shown that sulfated polymers will reversibly inhibit the action of various macrocationic substances having enzymic activities: hyaluronidase, ribonuclease and lysozyme. These proteins formed complexes with the anionic macromolecules and could be released or "activated" by the addition of other cationic agents such as sodium ion or protamine.

The formation of precipitates with optimal amounts of acidic macromolecules and macro-cationic substances has been compared to the specific precipitates resulting from antigen and antibody combinations and it has been noted that pro- and post-zones occur (10). Preliminary studies in this laboratory with free

diffusion of acidic polysaccharides against polymyxins in agar (as in the Ouchterlony technique) have shown that precipitates will form which have an appearance similar to those produced with antigen-antibody systems. It has been possible to show differences between certain of the polymyxins by means of their interaction with heparin and conversely it has also been possible to show differences between some of the connective tissue mucopolysaccharides by means of their interaction with polymyxin B. It is of interest that the similarities and differences noted amongst the polysaccharides in the latter instance are comparable to the previously reported differences and similarities of these polysaccharides as to their neutralization of polymyxin B and other cationic agents (4).

The demonstrated "anti-toxic" effect of heparin is achieved in a similar manner to that of antitoxin, *i.e.*, by formation of a complex with the toxic agent. However, this activity of heparin is not restricted to a single species of toxic agent by the high degree of specificity required for interaction in the toxin-antitoxin system. These properties of heparin support the idea that acidic mucopolysaccharides of connective tissue may play an important role in detoxification of certain noxious agents in the tissues. In this regard, previous work has demonstrated that heparin complexes are rapidly ingested by cells of the connective tissue where they are temporarily stored as cytoplasmic granules (4). The fate of the ingested material is not known, but the granular material disappears within 24 to 48 hours. In the present studies, the persistence of antibiotic activity in mice given 40 μg/gm dose of neomycin (table 2, C), of which at least one-half was in complex with heparin, would suggest that a significant portion of the heparin-bound drug was released from the complex during the 5 hour experimental period. Obviously, the rate of release was sufficiently slow as to not exceed the tolerance of the tissues for this substance. Since intravenously administered heparin has been readily detected within connective tissue cells within 2 hours after injection (8), it is probable that dissociation of the neomycin-heparin complex occurred intra- as well as extracellularly and rather improbable that the prolonged activity of neomycin was due to persistence of neomycin-heparin complexes in the circulation.

The release of neomycin from heparin may be analogous to

the liberation or "activation" of an endogenous bacteriocidal substance in the tissues and it is of interest that some antibacterial substances form complexes with acidic macromolecules *in vitro* (13). If these agents existed in similar complexes with heparin or heparin-like substances in the tissues, the displacement of these factors during an infectious process by toxic agents produced by the pathogen or by injured cells of the host could result in neutralization of the toxic substances with concomitant "activation" of substances capable of aiding other host mechanisms in the inhibition or elimination of the pathogen. Therefore, on the basis of the foregoing speculations concerning the function of heparin in the tissues, the degranulation of mast cells with consequent delivery of heparin into or in proximity of the site of injury could be a significant factor determining resistance in the earliest stages of an infectious process as well as to other types of injurious stimuli.

The observation in the present study that heparin could enhance the prophylactic value of neomycin in mice infected with a highly virulent strain of *Staphylococcus aureus* may have some clinical applications. However, it should be noted that the neutralization of acute toxic effect of neomycin shown in these studies was concerned only with the effects of a single lethal intravenous dose of neomycin and no attempt was made to determine the effect of heparin on toxicity resulting from prolonged administration of this drug. Dolowitz (3) has reported that heparin enhances the clinical usefulness of topical therapy with this group of antibiotics. Unfortunately, no toxicity controls were run with his patients and the data presented were not sufficient for evaluation of this aspect of the problem. On the other hand, Carter has followed up our initial studies at Utah on polymyxin B and heparin (14) and has found that although heparin neutralizes the toxic effects of polymyxin B, it does not appear to interfere with the antibiotic activity of this drug against either *Klebsiella pneumoniae* or *Pseudomonas in vitro* or *in vivo* (2).

Summary

The suggestion that heparin may be an important factor in natural resistance was discussed. Data was presented to show that heparin can neutralize the toxic effects of several antibiotic

agents. It was observed that the maximal dose of neomycin tolerated when administered in complex with heparin is greater than when administered without heparin. This dose of neomycin heparinate was more effective in protecting mice against the lethal effects of a virulent strain of *S. aureus* than was the maximally tolerated dose of neomycin without heparin.

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CARDIAC GLYCOGEN IN MONKEYS AND HUMAN SUBJECTS

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——Primate Cardiac Glycogen

The functional significance of glycogen in the myocardium is not known with certainty. It is well-established that, as in other tissues[3,20,10], glycogen of the heart may be divided into two fractions one of which is unbound and extractable with cold trichloracetic acid while the other is protein bound[10,20,22]. Despite differences in the effect of certain factors on the storage of glycogen in the myocardium and in skeletal muscle there is increasing evidence that in both sites glycogen subserves a reserve energy function. The literature is replete with data on the glycogen content of the myocardium of common laboratory animals under a variety of conditions, but is singularly devoid of similar data on the hearts of primates. The reason for this, particularly as regards the human heart, is apparent since glycogen disappears very rapidly from the heart if it is allowed to beat to a standstill against resistance.[9] However, with the advent of open cardiac surgery, as it becomes increasingly important to learn the amount, behavior, and significance of cardiac glycogen it also becomes practicable to obtain samples of the human myocardium in an approximately normal state. This report concerns the glycogen content of a small series of the four chambers of the monkey myocardium and of the human right atrium. Procurement of samples of the human ventricle will doubtless be realized in time, but meanwhile one can surmise approximately what the glycogen content of these areas may be from data on the distribution of glycogen in different areas of the myocardium of other species. Therefore, the distribution of glycogen in various portions of the dog myocardium is also presented for comparative purposes.

Methods

Human Myocardial Specimens. In patients undergoing corrective heart surgery the usual preoperative fasting period of 12–16 hours was enforced. Patients were premedicated with Demerol and some belladonna derivative.

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Anesthetic agents selected from sodium pentothal, ether, nitrous oxide, and fluothane, and adjuncts, oxygen and succinylcholine, were used in inducing analgesia and amnesia. Endotracheal intubation was instituted in each case allowing a completely patent airway and the prevention of any significant hypoxia was corroborated by normal arterial and venous oxygen saturations, electroencephalographic and electrocardiographic tracings. Following thoracotomy and preliminary great vessel dissection the right auricular tip was removed, the specimen blotted free of excess blood, coarsely macerated, immersed in 30% potassium hydroxide and heated in a water bath. The procedure from interruption of the blood supply of the auricular tip to the heating of the specimen was accomplished within 40 to 60 seconds.

Monkey Myocardial Specimens. Four mature male Capuchin monkeys weighing from 4½ to 6½ pounds were fasted for 22 hours following which they were anesthetized using pentobarbital sodium, 30 mg./kg. Some resistance on the part of the animals was encountered during anesthetization and was presumed to be associated with epinephrine and norepinephrine release from the adrenal medulla. A sternal splitting procedure was used and specimens of the right and left auricles and right and left ventricles were obtained. Additional specimens of liver and right gastrocnemius muscle as well as blood samples for glucose determination were obtained. In obtaining the heart specimens precautions were taken to prevent systemic or regional hypoxia and all tissue samples were handled as indicated above.

Canine Myocardial Specimens. Fifteen adult mongrel dogs of both sexes weighing from 16 to 45 pounds were fasted for 24 hours. Anesthetization was accomplished using pentobarbital sodium (35 mg./kg.). Immediately upon anesthetization a sternal split was performed, the pericardium incised, and samples taken successively from the right atrial and left atrial appendages and the right and left ventricles. Samples of blood for glucose determination, liver, and skeletal muscle of the right leg were also obtained. All specimens were handled as described above.

Tissue glycogen analyses were made using the method of Goode, Kramer, and Somogyi.[11] The determination of glucose obtained from hydrolysis of the glycogen was made by the method of Shaffer and Somogyi.[21] The blood sugars were estimated using the Hagedorn-Jensen[14] method.

Experimental Results

The glycogen content of the various portions of the myocardium and the other tissues of the animals studied is recorded as milligrams of glucose per 100 grams wet weight of tissue.

In Table 1 are presented the data on the glycogen content of human right atrial appendages obtained at operation on 9 individuals with congenital or acquired heart disease. The average glycogen content in this series was 793 mg. per 100 grams wet weight of tissue. The range varied widely from 479 to 1121 mg. per 100 grams of tissue.

In Table 2 are presented the data obtained on analysis of various tissues of the monkey. It will be noted that the glycogen content of the various portions of the myocardium is considerably lower than found in the dog (Table 3). It will also be noted that

TABLE 1

Glycogen content of the human myocardium
(patients fasted 12–16 hours)

Patient no.	Sex	Age	Diagnosis	Right auricular glycogen* (mgms/100 gms wet weight tissue)
1	Female	4 years	IASD + PS	761
2	Male	7 years	IVSD	1121
3	Male	10 years	IASD (Ost. 1°)	899
				(755)†
4	Male	16 years	AS	817
				704+
5	Male	17 years	MI	734
				1028+
6	Male	18 years	ISVD + Inf. PS	838
7	Female	22 years	IVSD c 3° HB	757
8	Female	36 years	IASD	586
9	Male	44 years	MI + MS	479
				793 ± 55

* Expressed as glucose.
† Second right auricular specimen taken at atrial closure following perfusion. Sample tissue partially separated from blood supply by the purse-string suture for 45 minutes.
+ Additional myocardial samples.

TABLE 2

Glycogen of the myocardium and other tissues of the monkey
(4 male Capuchin monkeys fasted 22 hours)

Tissue	Tissue glycogen* (mgms/100 gms wet weight tissue)
Right atrium	465 ± 17+
Left atrium	457 ± 33
Right ventricle	333 ± 19
Left ventricle	317 ± 43
Liver	568 ± 132
Skeletal muscle (Gastrocnemius)	563 ± 78
Blood glucose	107.3 mgm % ± 9.7

* Expressed as glucose.
+ S.E. $= \sqrt{\frac{\Sigma d^2}{N(N-1)}}$

the glycogen content of the atria is significantly greater than that of the ventricles. The glycogen concentrations in the gastrocnemius muscle and the liver are not remarkable. The blood glucose concentration is somewhat greater than one would expect in animals fasted for 22 hours.

The glycogen content of the tissues obtained from the dog is presented in Table 3. It is seen that the highest myocardial glycogen values are found in the left atrium and the right ventricle. Here again the average concentration of glycogen of the atria is greater than that of the ventricles. The glycogen content of the skeletal muscle and the blood glucose levels are within expected ranges for this species.

Discussion

Human myocardial glycogen values reported in the literature have been determined on postmorten specimens[6,18,24]. The authors are not aware of reported glycogen values determined in antemortem specimens of human hearts. The data presented indicate that the concentration of glycogen stored in the human atrium is not greatly different from that of the dog. One factor of uncertainty is that of age combined with a 12–16 hour fast. Cardiac glycogen storage increases with fasting and the rate of increase bears some relation to the size and age of the animal. The human

TABLE 3

Glycogen of the myocardium and other tissues of the dog

(15 dogs: 9 males, 6 females fasted 24 hours)

Tissue	Tissue glycogen* (mgms/100 gms wet weight tissue)
Right atrium	698 ± 58+
Left atrium	955 ± 69
Right ventricle	813 ± 58
Left ventricle (apex)	731 ± 50
Liver	2208 ± 334
Skeletal muscle	620 ± 46
Blood glucose	94.6 mgm % ± 3.7

* Expressed as glucose.

+ S.E. $= \sqrt{\frac{\Sigma d^2}{N(N-1)}}$

subjects with two exceptions are children and young adults in whom the effects of fasting would become apparent more quickly than in more mature adults. However, it is doubtful that in these subjects a fast of 12 to 16 hours is long enough to cause much increase in cardiac glycogen. Nevertheless, in this series there is an apparent trend such that the higher glycogen concentrations observed in the human atria are found in the younger subjects.

It was formerly held that glycogen of the myocardium was a rather stable constituent whose function was primarly that of a reserve which could be mobilized under unfavorable conditions. More recently, however, by the use of isotopically labeled glucose in studying the metabolism of the isolated beating heart it was found that plasma glucose may not only serve substantially as a direct source of cardiac energy, but it may be rapidly incorporated into cardiac glycogen. From 5 to 8% of the glycogen was replaced per hour by labeled glycogen indicating that cardiac glycogen may serve a more directly functional and dynamic role in cardiac metabolism.[8] When aerobic conditions prevailed glucose, if available, was the preferential substrate[23]. This apparent dynamism of glucose and glycogen in the myocardium in addition to the factors of body size and age is probably responsible for the variability seen in the determined values of glycogen in the human right atrium.

Several patients had been under treatment with cardiac glycosides for varying periods of time prior to surgery. Animal studies have yielded divergent results as to the effect of cardiac glycosides on cardiac glycogen. Reed and Kelsey[19] in work on young rats found no change or slight increases in cardiac glycogen with the administration of Digoxin (0.2 mg./kg.). With older animals receiving the drug there was a decrease in the TCA extractable glycogen fraction and a corresponding increase in the bound glycogen fraction, the total glycogen content being unchanged. Blain *et al*[1] reported no change in human myocardial metabolism with therapeutic doses of lanatoside-C. It is, therefore, assumed that the variation in cardiac glycogen in this series cannot be attributed to this medication. As the medication in each patient prior to and during surgery was essentially uniform for age and weight, the influence of these agents in relation to glycogen variability can be discounted.

Rather wide variability in the glycogen content of the various

portions of the dog heart are in evidence. Similar marked variations have been previously noted by Boyle and MacDonald[7] who failed to state if their animals were fasted. This factor may be of importance since it is quite possible that fasting may affect unequally glycogen storage in the various portions of the heart.

Speculation on a possible relationship between the work performed by various portions of the myocardium and the distribution of glycogen is of interest. It has been shown[5] in the rat that hypertrophy of the heart induced by increasing the workload of the left ventricle by experimental aortic coarctation is accompanied by an increase of glycogen predominantly in the unbound fraction. Data from this laboratory[15] regarding glycogen concentration in the myocardium of hypertensive rats show a similar finding. If this factor were responsible for variation in normal hearts one would expect the content of the left ventricle to exceed that of the right. Moses[16] reported a trend towards such a distribution of glycogen in the ventricles. However, the reverse circumstance appears to be the case in the present data on the dog, whereas, we find no difference in the glycogen content of the right and left ventricles of the monkey. In both the dog and the monkey we find the mean glycogen concentration of the atria to exceed that of the ventricles, thus one could hardly conclude that in the normal heart the distribution of glycogen stores is related to the workload.

Additional factors may be mentioned as potentially related to the distribution of glycogen in the myocardium. The first of these is the relative amount of connective tissue in the various portions of the heart. It might be expected that lower concentrations of glycogen would be found in those regions where the connective tissue makes up a larger fraction of the myocardium. It is known that the highest concentration of connective tissue in the heart is found in the interatrial septum[17], and histologically the walls of the atria appear to contain a higher percentage of connective tissue than do the walls of the ventricle. Further, in our experience the atria require a considerably longer period in hot 30% potassium hydroxide to be solubilized than do ventricular specimens, probably denoting a greater concentration of connective tissue in the atria. These observations might lead one to expect a lower concentration of glycogen in atrial than in ventricular tissue. The reverse was found to be the case. A second

factor concerns the relative distribution of coronary blood to the various portions of the heart. Although the relative blood flow in the right and left coronary arteries of the anesthetized dog has been measured[13], the relationship between the regional perfusion volume, tissue mass and workload remains obscure and may subsequently be found to be a primary determinant of cardiac glycogen distribution. It is conceivable that in the thin walls of the atria equilibration of subendocardial tissue with luminal blood might relate to differences in glycogen in the walls of the right and left chambers.

Hormonal effects on the distribution of cardiac glycogen are unknown. We have previously mentioned the probable release of adrenergic agents in the monkey during anesthetization. This is thought to be a possible factor in the rather low concentration of cardiac glycogen found in these animals. Bloom and Russell[4] have shown that moderate doses of epinephrine or norepinephrine subcutaneously were followed by an increase in cardiac glycogen. Large doses of epinephrine have been shown by Chang[9] to cause a reduction of cardiac glycogen by as much as 50% after a period of 25 minutes with recovery to normal glycogen levels in the subsequent half hour. It was during the first interval that the monkeys in this report were sacrificed. This adrenergic effect is thought to account for the blood glucose levels above those expected in animals fasted for 22 hours.

Summary

The glycogen content of the four chambers of the monkey myocardium and of human right atrium was determined. For comparative purposes similar studies on the dog myocardium were included. Liver and skeletal muscle glycogen and blood glucose levels in the monkey and dog were also estimated.

Rather wide variations in myocardial glycogen in all species were observed. The glycogen content in the human right atrium seemed to be related to age with younger individuals showing a generally higher glycogen content in the myocardium. There appeared in the human to be little influence on glycogen content of body weight or medications either prior to or during surgery.

In the monkey the myocardial glycogen content was lower than similar samples in the human or dog. The atrial glycogen

was significantly higher than that in the ventricles. These low values in cardiac glycogen were thought to be related to endogenous release of adrenergic substances related to anesthetization. The slightly elevated fasting blood glucose in the species tends to corroborate this belief.

In the dog myocardium the glycogen content varied markedly. The higher values were found in the left atrium and right ventricle. The average concentration of glycogen was greater in the atria than in the ventricles.

Factors related to variations in distribution of cardiac glycogen are discussed.

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CHROMOSOMAL CONDITIONS IN SOME HUMAN SUBJECTS WITH NONMALIGNANT DISEASES[a]

TAKAAKI ISHIHARA AND SAJIRO MAKINO[b]

The present article deals with a chromosome study on cells suspended in the peritoneal fluids taken from four patients with a diagnosis of liver cirrhosis and in those occurring in the pleural effusions derived from four cases of tuberculosis. Recent improvement of cytological techniques, particularly the development of the water pretreatment squash method, has made possible reliable investigation of the chromosomes in cells of a similar nature (Makino, 1957; Makino, *et al.*, 1959). A knowledge of chromosomes in cells from body fluids, such as peritoneal and pleural exudates which were produced by human disorders, may be valuable and significant for understanding the nature of cells in relation to malignancy. In the present materials, the chromosomes were analysed in comparison with those of both normal and malignant somatic cells. Though the research is not completely fruitful, publishing the results of the present investigation may not be uncalled for because research data in this field is very scarce at present.

Materials and Methods

The materials were derived from four patients who showed an accumulation of peritoneal exudate, and four patients who produced rich pleural effusion. Some of these cases were suspected in a preliminary clinical diagnosis of the disease with a question of malignancy. Some clinical and diagnostic data for each patient are summarized in Table 1.

Chromosomes were observed following the water pretreatment squash method (Makino, 1957). The peritoneal or pleural fluids with suspended cells were drawn directly from each patient into small bottles and centrifuged for 5 minutes at 700 r. p. m. in order to concentrate the cells. The droplets of cell suspension were placed on slides and a nearly equal volume of water was added to them and well mixed. After 5 to 10 minutes the samples were stained with acetic dahlia for 5 to 10 minutes. Squashing was done by applying an even finger pressure on the coverslip.

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Case Records

Clinical data in brief are shown in Table 1.

Cases 1–4: Each patient designated as cases 1 to 4 showed an accumulation of peritoneal fluids. Clinical examinations led to a diagnosis of cases 1 and 4 as liver cirrhosis, while the diagnoses of cases 2 and 3 in preliminary examinations were suspected of being due to hepatoma or to liver cirrhosis.

Cases 5–8: The patients referred to as cases 5 to 8 produced a rich amount of exudate in their pleural cavities. In preliminary pathological examinations of the fluid of cases 5 and 8 a malignancy or a tuberculous process was suspected, though further detailed examinations found the patients to suffer from pleural tuberculosis. Cases 6 and 7 were diagnosed as typical pleurisy.

Chromosomal Conditions

The chromosomal conditions were studied in cases 1 to 8 with the cells occurring in peritoneal or pleural effusions. The mitotic rate of cells suspended in the effusion was very low in every case; for instance, it was less than 0.005 per cent in cases 1 and 2.

Number of chromosomes: Because of very low mitotic frequency, chromosome counts were made only in a rather small number of metaphase plates which allowed exact examination. Tables 2 and 3 furnish the results of chromosome counts.

Reference to the tables indicates that the chromosome counts of cells fell in a diploid range for every case examined. Especially

TABLE 1

Some clinical data on the eight cases studied

Case no.	Effusion	Diagnosis	Age	Sex
1	Peritoneal	Liver cirrhosis	60	♀
2*	Peritoneal	Liver cirrhosis	60	♂
3*	Peritoneal	Liver cirrhosis	81	♂
4	Peritoneal	Liver cirrhosis	32	♀
5*	Pleural	Tuberculosis	35	♀
6	Pleural	Tuberculosis	22	♂
7	Pleural	Tuberculosis	21	♂
8*	Pleural	Tuberculosis	52	♂

* Cases 2, 3, 5 and 8 were diagnosed as suspicious in preliminary clinical examinations with a question of whether or not the effusions were of cancerous origin.

TABLE 2

Distribution of chromosome numbers in eight cases under study

Case no.	Chromosome number											Total	Percent of $2n$ (46)
	39	41	42	43	44	45	46	47	48	49	50		
1	.	.	.	1	1	.	26	.	1	1	.	30	86.6
2	1	.	.	1	.	1	16	1	.	.	.	20	80.0
3	.	.	.	.	1	1	11	.	.	.	.	13	84.6
4	.	.	.	.	.	1	5	.	.	.	.	6	83.3
5	.	.	.	.	.	.	6	.	.	1	.	7	85.7
6	.	1	.	.	.	.	22	1	.	.	1	25	88.0
7	.	.	.	.	.	.	4	1	.	.	.	5	80.0
8	.	.	.	.	.	.	5	.	1	.	.	6	83.3

TABLE 3

Distribution of chromosome numbers in four samples obtained in case 6

	Sampling (date)	Chromosome number										Total	Per cent of $2n$ (46)
		41	42	43	44	45	46	47	48	49	50		
I	(Sept. 2)	.	.	.	.	.	3	1	.	.	.	4	75.0
II	(Sept. 7)	1	.	.	.	.	5	.	.	.	.	6	83.3
III	(Sept. 30)	.	.	.	.	.	11	.	.	.	1	12	91.6
IV	(Oct. 9)	.	.	.	.	.	3	.	.	.	.	3	100.0
	Total	1	.	.	.	.	22	1	.	.	1	25	88.0

remarkable was the fact that the cells showing 46 chromosomes occurred in the highest frequency in all cases, being more than 80 per cent in the metaphasic cells observed (cf. Table 2). In case 6, four samplings were made at different intervals during a period of 37 days as shown in Table 3, and the cells having 46 chromosomes were found with the highest frequency in every sample. A few cells showing a numerical deviation from 46 were distributed in a range between 39 and 50 (Table 2). This might have been due either to mitotic aberrations such as non-disjunction and anaphase elimination of chromosomes, or to technical accidents. Polyploid cells were found exclusively in case 6, being only about 1 per cent in frequency.

Following the new findings on human chromosomes by Tjio and Levan (1956), extensive investigations with the use of improved techniques and of a variety of tissues have supplied similar evidence that forty-six is the correct number for man. This

is generally accepted for different human races (Ford and Hamerton, 1956; Ford, *et al.*, 1958; Tjio and Puck, 1958; Makino and Sasaki, 1959, 1960; Chu and Giles, 1959; Levan and Hsu, 1959). In reference to the reported evidence, it is possible that the vast majority of cells occurring in the peritoneal and pleural exudates taken from eight patients in this study contained the normal chromosome number for man.

Morphology of chromosomes: Recent investigations have shown that the normal 46 chromosomes of man consist of twenty-two pairs of autosomes and one sex-determining XY-pair. The individual chromosomes of the normal somatic complement have been identified recently by several authors (Tjio and Levan, 1956; Ford, *et al.*, 1958; Tjio and Puck, 1958; Chu and Giles, 1959; Makino and Sasaki, 1959; Levan and Hsu, 1959) on the basis of their size and the position of their centromeres.

In the present study, the 46 chromosomes with cells derived from 8 cases were also morphologically analysed on the basis of size and shape, according to a system of nomenclature proposed by the Human Chromosome Study Group (April, 1960, Denver, Colorado). They were arranged as nearly as possible in descending order of size.

In the majority of cells, however, matching the chromosomes in supposed homologous pairs was a task producing considerable difficulty since some chromosomes were quite indistinguishable from one another in size and shape, and remained unidentified because of a deformation caused by some technical accidents. Regardless of these difficulties, the chromosomes were morphologically comparable with those reported by recent authors for the normal somatic cells of man. The results may be understood by referring to Figs. 1 to 15.

Some mention should be made of the sex chromosomes. According to recent studies by Tjio and Puck (1958); Ford, *et al.* (1958); Chu and Giles (1959); Levan and Hsu (1959) and others, there is a general agreement that the X chromosome is one of the larger medium-sized submedian chromosomes, while the Y is one of the smallest acrocentric chromosomes in the whole complement. In an approximation to the above view, Makino and Sasaki (unpublished) quite recently have reached the conclusion that the X is one of the larger medium-sized submedian chromosomes which seems to rank in the descending

order of size between the 6th and 7th autosomes, while the Y chromosome is recognized as one of the smallest acrocentric ones, nearly similar to or slightly larger than the autosome number 21. Generally the X chromosome is quite difficult to identify, even with a close analysis of the whole ideogram, whereas the occurrence of the Y is easily recognized due to the fact that there are five acrocentric chromosomes of prominently small size in the male complement, one of which is to be the Y, while in the female complement the number of the corresponding acrocentric elements is four, the Y being absent. By virtue of these features, therefore, the sex of the subject from which the tissue has been derived is rather simply recognized.

As shown in Table 1, cases 1, 4 and 5 are female, while cases 2, 3, 6, 7 and 8 are all males. The cells derived from each of the former cases clearly showed four acrocentric chromosomes of outstandingly small size as a designation of female sex (Figs. 8, 9, 12 and 13). Those of the latter cases which are male specimens are distinguishable from the former by virtue of the occurrence of five outstandingly small acrocentric chromosomes of corresponding size, one of which is to be the Y (Figs. 10, 11, 14 and 15). It was difficult to identify the X in these cells.

In conclusion it is evident that, from the results shown in the present study, the cells occurring in peritoneal exudates caused by liver cirrhosis and in pleural effusions produced by tuberculosis were found to contain chromosomes which showed no visible difference not only in number but also in morphology from those of the normal tissue cells of man.

Discussion

Since the chromosome investigations of human cancers and leukemias have recently used improved techniques (Hsu 1954; Hansen-Melander, *et al.*, 1956; Ising and Levan, 1957; Makino, Ishihara and Tonomura, 1959), a knowledge of the normal chromosome constitution of man is significant in differentiating between malignant cells and nonmalignant processes. General conclusions recently obtained by modern cytologists working with human neoplasms are that, though the number of chromosomes of human tumors exhibits a pronounced variation, there is present in each tumor a stem line/or lines of tumor cells which

occur most frequently with a characteristic mode/or modes different from the normal one and with a specific chromosome ideogram/or ideograms, clearly distinguishable in both number and morphology from those of other tumors as well as from normal tissue cells. It is then evident that each tumor is provided with its own characteristic stem line chromosomes which clearly differ numerically as well as morphologically from those of the normal somatic cells. Generally, most of the chromosomes of neoplastic cells differ in shape and/or size from those of normal cells because of structural and mutational alterations of individual chromosomes (Levan 1956, Makino 1957).

The chromosomal condition in tissue cells may be useful as a diagnostic criterion or it may be helpful in the recognition of malignant from nonmalignant cells. The cytological diagnosis of cancer has been thus far carried out with a variety of methods through the examination and identification of exfoliated cells in secretions of the female genital tract, of the stomach, and in other body fluids such as sputum, pleural and peritoneal exudates. Exfoliated cells are usually diagnosed by a common criterion of cytological patterns as represented by the presence of nuclear abnormalities such as enlargement, hyperchromasia, multinucleation, irregularity of shape, etc. (Papanicolaou, *et al.*, 1947; Rufin, 1956). The application of such cytological methods for the diagnosis of malignancy has been occasionally handicapped by poor preservation of the material. In recent years the improvement of simple squash techniques, particularly of the water pretreatment squash method, has made possible reliable chromosome research on cells suspended in body fluids of both animals and man. Makino, *et al.* (1959) undertook chromosomal analyses of cells occurring in peritoneal or pleural exudates taken from several tumor patients and found the stem line chromosome patterns characteristic to each tumor and different from those of the normal tissue. For instance, a mammary carcinoma was characterized by stem lines having 75 chromosomes, while a gastric carcinoma showed three types of stem cells, two at hypodiploidy (42 and 45) and one at hypotetraploidy (84).

In the present investigation, the chromosomes were observed in cells from the peritoneal effusions of four patients who were diagnosed as having liver cirrhosis, and in those suspended in the pleural exudates taken from four patients with tuberculosis

(Table 1). Some of these cases were diagnosed as suspicious in preliminary clinical examinations with a question of whether or not the effusions were of cancerous origin. The vast majority of cells taken from the eight cases showed the chromosome constitution to be identical to the normal somatic complement both in number and morphology, with a frequency of over 80 per cent of observed cells in every case (Table 2). These results served to clarify the diagnosis of suspicious cases. Further, on the basis of the ideogram analysis the identification of the sex was available in the cells under observation because of the existence of the Y chromosome in male cells.

The data here presented suggests that the cells occurring in peritoneal or pleural effusions produced by tumor patients furnished the chromosome pattern characteristic of tumors, while the cells suspended in the exudates transferred from nontumorous patients have shown the normal chromosome pattern. By virtue of this evidence, karyological data would be worthy of consideration in diagnostic cytology.

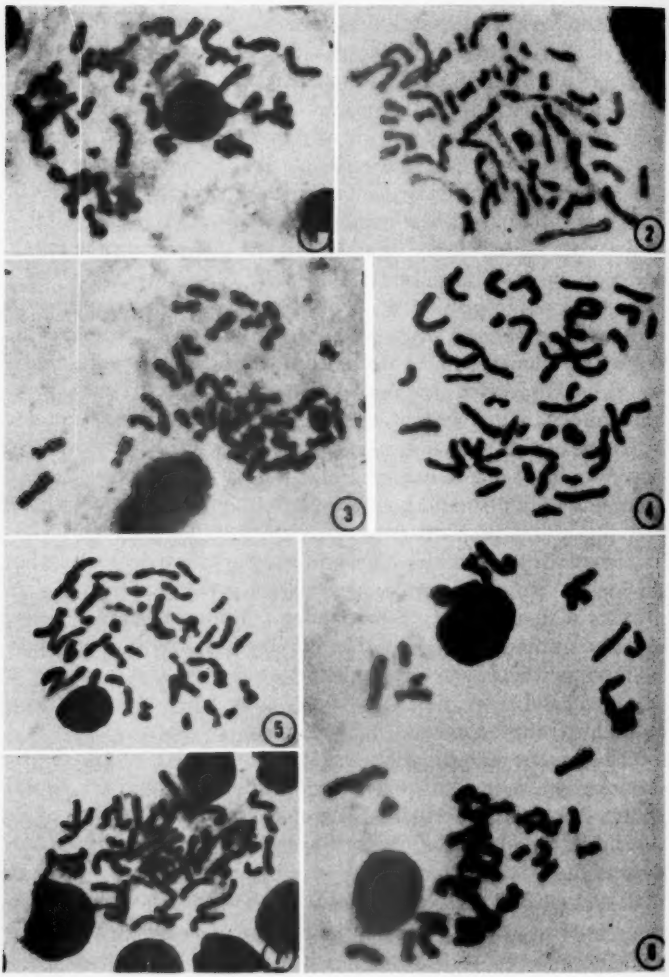
Some mention should be made here that certain tumors have been found in which the stem line chromosomes are identical in number with those of the normal cells. For instance, an ovarian carcinoma had 46 chromosomes with a general morphological likeness to the normal chromosomes (unpublished). However, closer examination revealed that the likeness was merely superficial, since the tumor chromosomes indicated several signs of morphological alterations by which they were clearly distinguishable from the normal chromosomes.

The present investigation has not included a study of the tissue origin of the cells occurring in the peritoneal or pleural fluids. This should be the subject of future work because of its clinical and pathological importance in connection with an understanding of the nature and type of the diseases.

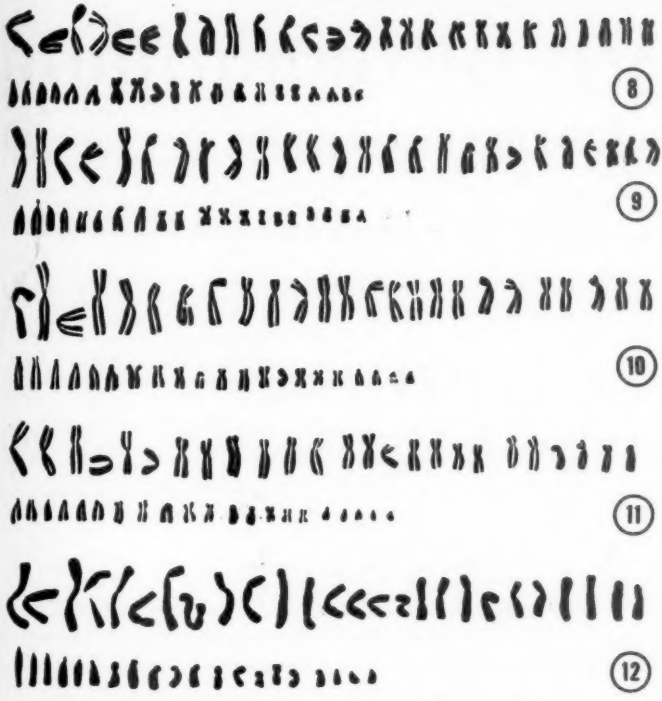
Karyological data from various types of diseases should be accumulated in order to provide basic information significant for clinical and pathological diagnoses of various human disorders.

Summary

Chromosomes were studied in cells suspended in the peritoneal exudates taken from four patients with liver cirrhosis and in the



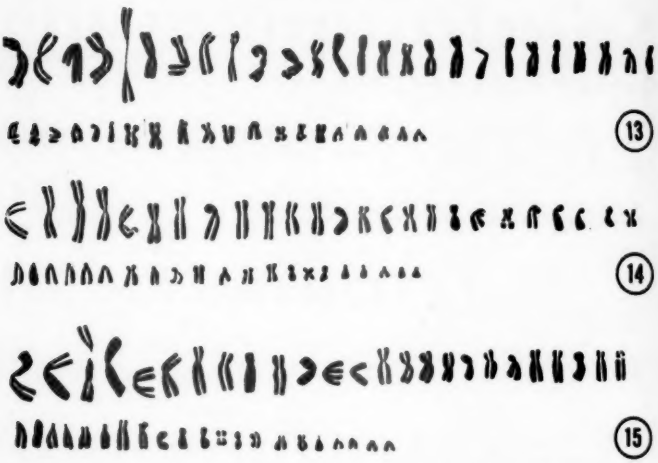
FIGS. 1–7. Photomicrographs of chromosomes in cells from peritoneal and pleural effusions. 1200X. FIGS. 1–4. Cells from peritoneal effusions taken from patients with liver cirrhosis (cases 1, 2, 3 and 4, respectively). FIGS. 5–7. Cells from pleural exudates taken from patients with tuberculosis (cases 5, 6, and 7, respectively).



FIGS. 8–12. Ideogram analyses of chromosomes from peritoneal effusions. FIGS. 8 and 9 from case 1 (♀). FIG. 10 from case 2 (♂). FIG. 11 from case 3 (♂). FIG. 12 from case 4 (♀).

pleural effusions furnished by four patients having tuberculosis, by way of comparison with the somatic chromosomes of both normal and malignant cells. Consistent counts showed the normal diploid number of chromosomes to be 46 in the vast majority of cells taken from the eight cases. The ideogram analysis has revealed that the chromosomes showed nothing visibly different from those of the normal tissue cells of man in many morphological respects. The cells from male specimens were chromosomally diagnosed on the basis of the existence of the Y chromosome.

The suggestion was made that the chromosome complex sometimes may be valuable as a criterion for the cytological diagnosis of malignancy.



FIGS. 13–15. Ideogram analyses of chromosomes from pleural exudates. FIG. 13 from case 5 (♀). FIG. 14 from case 6 (♂). FIG. 15 from case 8 (♂).

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TIME-DISTRIBUTION STUDIES OF SELENIUM-75 IN DOG SERUM PROTEINS*

KENNETH P. MCCONNELL, C. H. WABNITZ, AND D. M. ROTH

Previous studies *in vivo* (6) have demonstrated that when serum proteins from dogs injected 24 hours earlier with Se^{75} were resolved by paper electrophoresis, 60 per cent of the total radioactivity was found distributed equally between the alpha-2 and beta-1 globulin fractions. Smaller amounts, namely 13, 9, 11 and 7 per cent, were observed in the albumin, alpha-1, beta-2 and globulin fractions, respectively. It was the purpose of the current series of experiments to determine whether there was any quantitative difference in selenium binding in the various serum proteins as related to time. In order to obtain the desired information, dogs were injected with trace amounts of Se^{75} and the radioactivity of the serum protein fractions was determined at various time intervals. It was found within the first hour after administration of Se^{75} that the greatest radioactivity was present in the albumin fraction, after which there was a gradual decrease. The alpha-2 and beta-1 activity values were low immediately after administration of Se^{75} and increased with time, until a plateau was reached which was maintained for 14 weeks. The results were interpreted to indicate that selenium is bound to serum proteins in at least two different forms, and that albumin acts in the transport of selenium in the dog.

Methods

Dogs I and II were injected subcutaneously with $Se^{75}Cl_4$ which contained 1.8 mc. Se^{75} (15.4 mg. Se) and 1.63 mc. Se^{75} (3.27 mg. Se), respectively. Dogs III and IV each received 0.78 mc. Se^{75} (0.05 mg. Se) as $H_2Se^{75}O_3$. Samples of blood were taken from dogs I and II and analyzed at various intervals during 48 hours (Fig. 1), while blood from dogs III and IV (Table 2) was assayed from one to 14 weeks. Electrophoretic analyses were performed in a Spinco Model R, Durrum-type paper electrophoresis apparatus. Separations of serum samples were carried out for 16 hours at 2.5 MA. in veronal buffer, ionic strength 0.05, pH 8.6 on Whatman 3 MM paper strips, which were oven dried at 120° to 130°C for 30 minutes, then dyed with bromphenol

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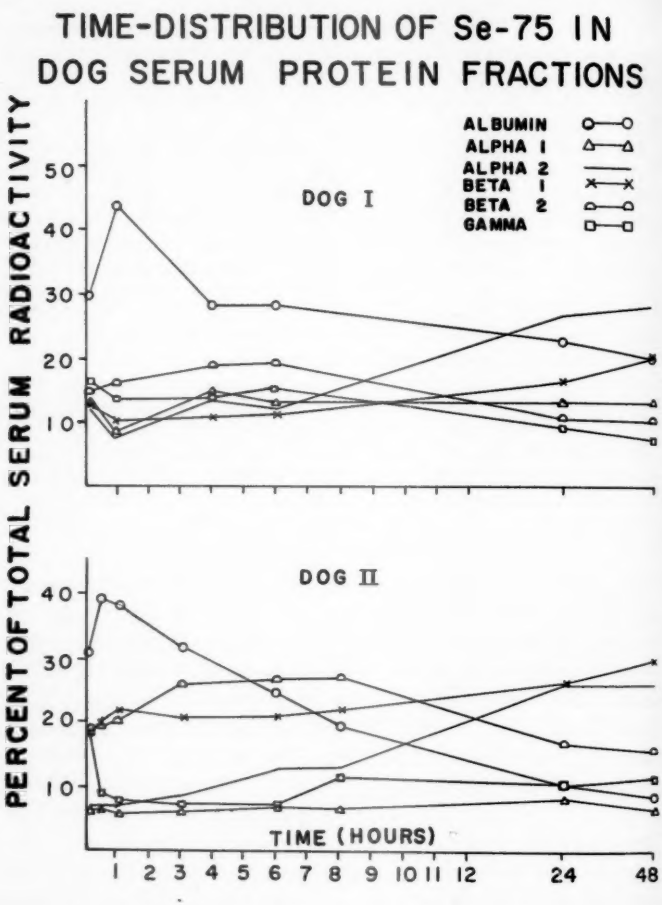
blue. The Spinco Analytrol calibrated recording densitometer and automatic integrator was used to evaluate the relative amounts of protein in the stained material separated electrophoretically.

Total serum protein radioactivity values were obtained in the following manner: (1) the stained and dried electrophoresis papers (3 × 30 cm.) were rolled tightly, (2) placed in the bottom of a 1¼ × 12½ cm. test tube, and (3) the tube was placed in a Berkeley well-type scintillation detector (sodium iodide crystal) for radioactivity assay. Values obtained in this manner represented total serum protein Se^{75}. The various serum protein fractions were cut from the electrophoresis paper and assayed separately for radioactivity as indicated above. The results in Fig. 1 are expressed as the per cent of the total serum count found on the electrophoresis paper in the various protein fractions. In dog II, the serum protein was precipitated by the standard trichloroacetic acid method, and the resulting precipitate assayed for radioactivity to give the protein-bound Se^{75}. Serum protein-bound selenium was also obtained by dialysis of serum in Visking casing against running tap water for 24 hours, and these values were compared with the trichloroacetic acid precipitated protein-bound selenium values. Sodium heparin (1,000 U.S.P. units per 10 ml. whole blood) was used as an anticoagulant in the experiment (Fig. 2) in which the concentration of Se^{75} in plasma in relation to time was studied.

Results and Discussion

Figure 1 shows the distribution of Se^{75} at different time intervals in the various protein fractions for both dogs I and II. Within 10 minutes after subcutaneous injection of $Se^{75}Cl_4$, about 30 per cent of the total count was present in the albumin fraction from both dogs. Values for the remaining serum protein fractions, *i.e.*, alpha-1, alpha-2, beta-1, beta-2 and gamma globulin, ranged from 6.1 to 19.2 per cent. The albumin value in dog II rose to 39.4 per cent in one-half hour, while in dog I at the one-hour interval the value was 43.6 per cent. Thereafter, in both dogs a similar pattern was observed in which the albumin percentage gradually decreased with time up to 48 hours. The final concentrations were one-half and one-quarter of the peak concentrations in dogs I and II, respectively. At the 10-minute interval, alpha-2 and beta-1 percentages of the total count were respectively 12 and 13 per cent for dog I, and 7 and 18 per cent for dog II. Afterwards there was a gradual increase in alpha-2 and beta-1 globulin values up to 48 hours, where the alpha-2 and beta-1 values were found to be respectively 28 and 20.3 per cent for dog I, and 26.4 and 30.3 per cent for dog II. Beta-2 fraction, which was at a low value at the 10-minute period,



showed a temporary rise in both dogs around the 3-hour interval and subsequently decreased below the initial value by 48 hours. The remaining serum fractions, *i.e.*, alpha-1 and gamma globulin fractions, remained essentially unchanged at a low level throughout the entire experiment.

Comparison of the dialysis and trichloroacetic acid methods is shown in Table 1. Protein-bound selenium determinations by the trichloroacetic acid and dialysis methods show that as soon as 10

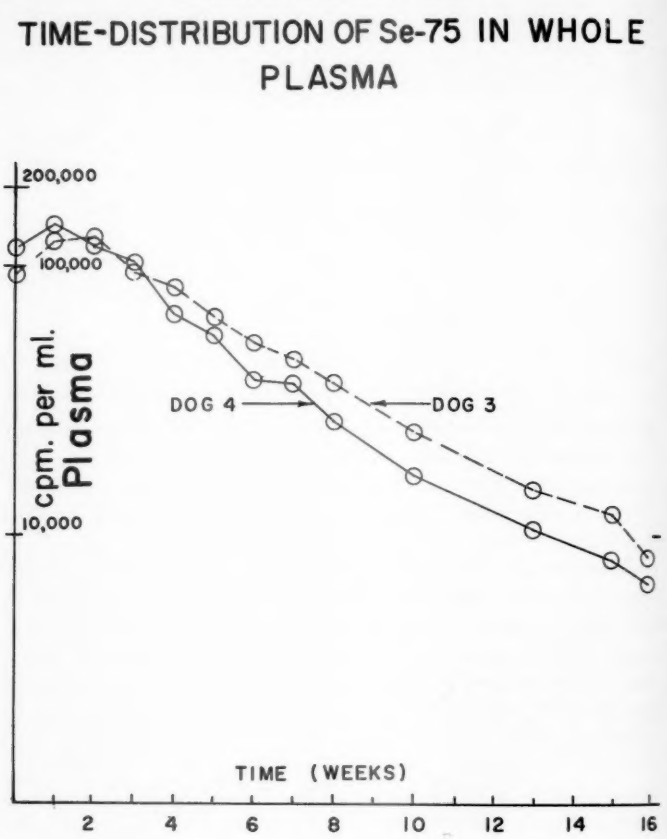
TABLE 1

Serum protein-bound Se^{75} in dog II

Time after injection	Activity of whole serum (c.p.m. × 10^3/ml.)	Protein-bound Se (percent total serum activity)	
		Trichloroacetic acid method	Dialysis vs. tap water
10 min.	36	69.7	54.8
½ hr.	322	. . .	58.8
1 hr.	409	78.4	62.4
3 hr.	392	74.8	68.8
6 hr.	316	80.4	68.7
8 hr.	286	82.7	75.5
24 hr.	177	85.4	85.7
48 hr.	141	93.8	95.4

minutes after administration of Se^{75}, 54.8 to 69.7 per cent of the total serum Se^{75} is protein bound, indicating a very rapid binding of selenium to protein. The percentage of protein-bound Se^{75} increases with time, and at the 48-hour interval values of 93.8 and 95.4 per cent are found. The non-precipitated Se^{75} in the trichloroacetic acid experiments and the dialyzable Se^{75} in the dialysis experiments are assumed to be inorganic selenium. In the intervals from 10 minutes to 8 hours the trichloroacetic acid method shows consistently higher protein-bound selenium values than those obtained by the dialysis method. These results suggest that the Se^{75} precipitated by trichloroacetic acid contains loosely bound as well as protein-incorporated selenium, indicating that selenium is bound to serum proteins in at least two different forms. It appears that selenium is first loosely bound to albumin, since at the 24-hour and 48-hour intervals (Fig. 1, dog II), the percentage of the total radioactivity in albumin is about one-quarter that of the peak concentration at the 0.5 hour-interval.

The per cent distribution of radioactivity in the serum proteins in dogs III and IV during the 14-week period was essentially the same as that obtained from dogs I and II at the 48-hour interval. Further, the per cent of the total count in the various protein fractions remained constant with minor variations from the first to the fourteenth week. It is of interest to point out that despite the fact that the Se^{75} concentration in plasma decreased with time (Fig. 2), the relative concentration in the various protein fractions remained fairly constant. These results are interpreted to



indicate that the rate of disappearance of Se^{75} from the various protein fractions after equilibrium is reached about the same for each serum protein. Protein-bound selenium as determined by the trichloroacetic acid method was 94.14 ± 0.63 per cent (dog III), and 97.34 ± 1.48 per cent (dog IV) of the total serum selenium during the 14-week interval.

In dog II, in order to estimate the amount of selenium bound to albumin and the variation of selenium content with time, certain calculations were made. Assuming the molecular weight of albumin to be 69,000 and using the value 1.2×10^6 c.p.m. per

TABLE 2

Distribution of Se^{75} in dog serum proteins during fourteen weeks

Dog	No. of determinations*	Radioactivity of serum proteins as percent of the total					
		Albumin	$Alpha_1$	$Alpha_2$	$Beta_1$	$Beta_2$	Gamma
		%	%	%	%	%	%
III	14	4.4±1.2	7.2±1.7	36.9±3.0	31.0±4.9	14.5±1.8	5.5±3.0
IV	14	3.6±0.6	5.5±1.0	31.2±3.5	39.1±4.2	17.1±2.4	3.2±2.8

* Distribution studies were made once a week for fourteen weeks.

μg. Se, the specific activity of a standard sample of Se^{75}, the amount of selenium in albumin was calculated. Expressed as gram-atoms selenium x 10^{-3} per mol. albumin, the values 0.25, 2.54 and 0.34 were found at 10 minutes, 1 hour and 48 hours, respectively.

Experimental evidence presented here indicates that after the injection of Se^{75}, albumin is the immediate acceptor of the greater part of serum selenium, and that over a period of time it is gradually released to, or bound by, other serum proteins, namely alpha-2 and beta-1 proteins. It is probable that albumin is involved with the transport of selenium directly or indirectly to more stable binding sites in blood and various tissues. These studies also suggest that there are at leat two different types of selenium binding to serum proteins. The albumin type of binding appears to be more labile than that of the alpha-2 or beta-1. The form in which selenium is incorporated in mammalian proteins is not definitely known. However, recent experiments (7) using tracer and chromatographic techniques have revealed that dog liver protein hydrolysates have at least three different fractions containing Se^{75}, one found in the cystine-selenocystine area, another in the methionine-selenomethionine area, and a third in the area of the leucines. Similar results have been obtained in unpublished experiments on dog plasma protein hydrolysates. The so-called loosely bound as contrasted to incorporated selenium is most likely an anion-protein combination. The *in vivo* mechanism of entry of radioselenium into the selenium analogues of the sulfur-containing amino acids, *i.e.*, cystine and methionine, has not been clearly established. The question arises whether the presence of selenium as selenocystine and selenomethionine indicates *de novo* synthesis of these compounds from inorganic

selenium. It is known that yeast and E. coli can synthesize the selenium analogues of methionine and S-adenosyl-methionine (11, 8, 9) from inorganic selenium. S-adenosyl-methionine acts as a methyl donor (3), as well as a donor of its aliphatic side chain in the biosynthesis of a variety of compounds (10). In the mammalian organism unconfirmed evidence demonstrated the formation of selenomethionine and selenocystine from inorganic selenium (7), and the enzyme systems capable of catalyzing the activation of methionine have been found in the tissues of many mammalian species (2, 5). If the same or similar reactions occur in dogs as in yeast and E. coli, *i.e.*, the formation of selenomethionine and activated selenomethionine ("Se-adenosyl-selenomethionine"), a possible explanation for the transfer of inorganic selenium to selenomethionine and selenocysteine may be as follows:

inorganic selenium → selenomethionine → seleno-adenosyl-methionine seleno-adenosyl-homocysteine → selenohomocysteine → seleno-cystathionine → selenocysteine.

It is of interest to point out that the phenomenon of differential binding of trace elements in serum with time as shown here for selenium has been observed with other trace elements, namely copper (4, 1) and cobalt (4). Although copper and cobalt are not related biologically to selenium, as far as is known they act in a similar fashion in serum time-distribution studies. It has been reported by Horst (4) that albumin in blood acts as a carrier for copper and cobalt. He observed that when $CuCl_2$ was administered intraperitoneally to mice, the copper was found in the albumin fraction immediately after injection, but was present chiefly in the alpha-2 globulin fraction after 2 hours. Cobalt administered as $CoCl_2$ was found principally in the albumin fraction after 45 minutes, and later was found in the alpha and beta globulins. The observation that selenium is bound first to albumin, and after an interval of time incorporated principally into specific globulin fractions, may be interpreted to indicate (as with copper and cobalt) that albumin acts in the transport of selenium.

Summary

After the administration of trace amounts of Se^{75} to dogs, distribution studies at different time intervals of Se^{75} in the

various serum proteins were made. Within the first hour after the subcutaneous injection of Se^{75} the greatest radioactivity was found in the albumin fraction, after which there was a gradual decrease. The alpha-2 and beta-1 radioactivity values were low immediately after administration of Se^{75} and increased with time, until a plateau was reached which remained relatively constant for 14 weeks. The results were interpreted to indicate that there are at least two different types of selenium binding in dog serum proteins, and that albumin acts in the transport of selenium in the dog. A possible mechanism of the entry of selenium into the selenium analogues of the sulfur containing amino acids is discussed.

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The technical assistance of Benjamin R. Larke is gratefully acknowledged.

URIDINE DIPHOSPHATE GLUCOSE AND URIDINE DIPHOSPHATE N-ACETYL-GLUCOSAMINE IN ERYTHROCYTES[a]

GORDON C. MILLS[b]

Previous studies using ion exchange analysis have shown that a wide variety of nucleotides are present in human erythrocytes (1, 2, 3, 13). Many of these nucleotides have been adequately characterized by previous workers. Bartlett (1) has presented careful studies characterizing adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), and triphosphopyridine nucleotide (TPN) of elution profiles. Diphosphopyridine nucleotide (DPN) has been identified by its absorption spectrum, by paper chromatography and as an adenine-containing nucleotide (3). DPN has also been identified by the absorption curve of its cyanide complex which has a distinctive absorption peak at 325 mμ (13). Guanosine triphosphate (GTP) has been identified by paper chromatography and by its absorption curves at pH 1, 7 and 11[c]. In addition, other workers have identified GTP by paper chromatography of the nucleotide and also by paper chromatography of the guanine which was liberated by acid hydrolysis of the nucleotide (3). The possibility still remains however, that there are other unknown components in the GTP peak. Recently, the cytosine diphosphate compounds have been characterized as deoxycytidine diphosphate choline, cytidine diphosphate choline, cytidine diphosphate ethanolamine and deoxycytidine diphosphate ethanolamine (12). The unknown component immediately following the ATP peak (Peak D of (13)) remains to be characterized (8). In addition, several nucleotide peaks (Peaks B and C of (13)) just preceding the ATP peak in the elution profile had not been adequately characterized previously. The suggestion was made that these components were uridine diphosphate glucose (UDPG) and guanosine diphosphate (GDP) (13). Although GDP is usually

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[c] G. C. Mills and D. F. Fincher, unpublished observations.

present, the amount is low and variable and uridine nucleotides make up the larger portion of these peaks.

The present study describes the isolation and characterization of two uridine diphosphate compounds which appear in this portion of the elution profile. Previous studies from this laboratory and also work by Bishop *et al.* (3) indicated that these compounds appeared in the elution profile at a position very close to that occupied by authentic UDPG. Other studies have shown, however, that UDPG and uridine diphosphate N-acetylglucosamine (UDPAG) are only partially separated using formic acid + ammonium formate as the eluant solution (4, 6, 7). In addition, various workers have shown that uridine diphosphate galactose and uridine diphosphate N-acetylgalactosamine are also present in mammalian tissues (7, 14, 18). As yet, neither the techniques of ion exchange or paper chromatography have successfully separated the two UDP-hexoses or the two UDP-acetylhexosamines. Consequently these four compounds must all be considered as possibilities in the identification of the uridine nucleotides of erythrocytes. The work described in the present study provides evidence to show that the two predominant uridine nucleotides of the erythrocyte elution profile are UDPG and UDPAG. Although UDP-galactose and UDP-N-acetylgalactosamine were not demonstrated in these studies, very small amounts of these components may still be present. Normal erythrocytes have the enzymes required for the interconversion of UDPG and UDP-galactose (10), and it may be presumed that, under certain circumstances at least, UDP-galactose would be present.

Materials and Methods

UDPAG, N-acetylglucosamine and UDPG dehydrogenase (Type III) were obtained from Sigma Chemical Company, UDPG was obtained from Mann Research Laboratories, Inc., and uridine monophosphate (UMP) and uridine diphosphate (UDP) were obtained from Pabst Laboratories. Darco G-60 activated carbon is manufactured by the Atlas Powder Company and was obtained from W. H. Curtin and Company.

UDPG was determined enzymatically with UDPG dehydrogenase as described by Strominger, *et al.* (17). Control studies were carried out using authentic UDPG which had an activity in this analysis equal to that shown by the UDPG of Strominger, *et al.* (17). The N-acetylglucosamine content of uridine nucleotides was determined by the reaction using p-dimethylaminobenzaldehyde as described by Reissig, *et al.* (16). Ribose analyses were carried out by the orcinol procedure using D-ribose as a standard (9).

The general procedures which have been utilized in anion exchange separation of acid-soluble phosphate compounds of erythrocytes have been described previously (12, 13). In each case, gradient elution has been used to provide a gradually increasing concentration of formate in the eluant solution. For the initial separation of uridine nucleotides from the blood filtrate (Fig. 1), a 3 cm. x 33 cm. Dowex 1-X4 column was utilized with a 500 ml. mixing reservoir which initially contained water. Elution was carried out with the following solvents in order: *1*, 2000 ml. of 0.5 N formic acid; *2*, 2000 ml. of 4 N formic acid; and 3, 2500 ml. of 4 N formic acid + 0.8 M ammonium formate. Approximately 13 ml. of eluent was collected in each fraction. For rechromatography of uridine nucleotides by the procedure of Hurlbert, *et al.* (9), elution was carried out using a 500 ml. mixing reservoir and a pH 5.0 ammonium formate buffer (1.0 M) in the upper reservoir. Approximately 10 ml. of eluent was collected in each fraction. A value of 9.9 was used in these studies as the millimolar extinction coefficient at 260 mμ for uridine compounds (5).

Paper chromatography was carried out using the ascending technique with Whatman No. 1 filter paper. The solvent moved about 18 cm. in each case (5 to 6 hours). The following solvent systems were used: (I) isobutyric acid—ammonium hydroxide—water, 66:1:33, (5, 12); (II) 95 per cent ethanol—1 M ammonium acetate (pH 7.5), 70:30, (5, 12, 14); (III) 0.1 M phosphate (pH 6.8)—ammonium sulfate—n-propanol, 100:60:2, (5), (IV) 95 per cent ethanol—1 M ammonium acetate (pH 9.0) saturated with sodium tetraborate, 70:30, (11, 12); (V) 95 per cent ethanol—1 M ammonium acetate (pH 3.8), 70:30 (5, 14). Solvents I, II, III and V were prepared as described in Pabst Circular OR-15 (5) and Solvent IV was prepared as described by Klenow and Lichtler (11). The position of the compounds on chromatograms was determined by examination under ultraviolet light.

Results

Ion Exchange Separation of Uridine Nucleotides. The nucleotides from 1 liter of human blood (2 donors) were separated on a Dowex 1-X4 anion exchange column as described above. The portion of the elution profile which contains uridine nucleotides is shown in Fig. 1. The fractions which contained uridine diphosphate compounds were pooled, the nucleotides were adsorbed on Darco G-60 and eluted with 50 per cent ethanol using the procedure of Pontis, *et al.* (15)[d]. The uridine nucleotides were absorbed on a 1 cm. x 21 cm. Dowex 1-X4 column and eluted with

[d] When these nucleotides are adsorbed from an acid solution, it is necessary to wash the Darco G-60 with a more alkaline solution prior to the elution with 50 per cent ethanol. With cytidine nucleotides, a pH 7.5, 0.1 M phosphate buffer was satisfactory; with uridine nucleotides, it was necessary to wash the Darco with 0.5 M Na_2HPO_4 (pH of about 9). In each case the excess phosphate was washed through the column with a small amount of water prior to elution with the 50 per cent ethanol. Recoveries of the nucleotides in these studies averaged about 80 per cent.

a pH 5.0 formate buffer using gradient elution as described by Hurlbert, *et al.* (9). The separation by this procedure of the uridine components into two major peaks, X_1 and X_2, is shown in Fig. 2. The fractions containing these components were assayed enzymatically for UDPG and these results are also shown in Fig. 2. It will be noted that the UDPG content of the X_2 peak as determined enzymatically corresponded very closely with the uracil content as calculated from the optical density readings at 260 mμ. The enzymatic analysis indicated that the X_1 peak contained no UDPG. When known uridine nucleotides were subjected to ion exchange chromatography using the same conditions, the peaks of UDPAG and UDPG corresponded with those of unknown components X_1 and X_2 respectively, and were well separated from the UMP and UDP peaks (Fig. 2). The small UMP peak which may be seen in the elution pattern of uridine nucleotides (Fig. 2) is probably due to the breakdown of a small amount of uridine diphosphate compounds during the isolation procedure.

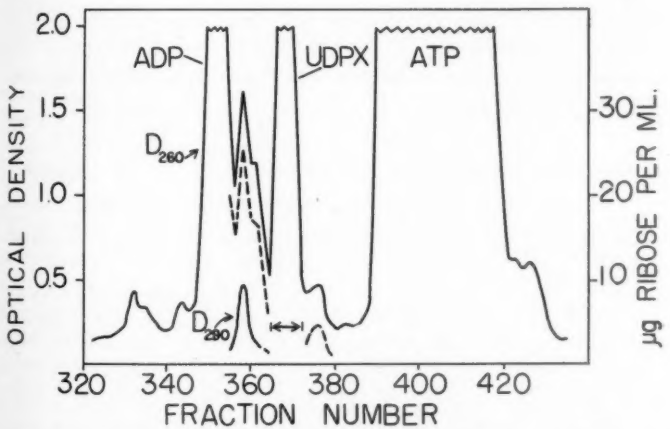

FIG. 1. An elution profile showing the ion-exchange separation of uridine nucleotides (UDPX) from 1 liter of blood. A 3 cm. x 33 cm. Dowex 1-X4 column was used and the nucleotides were eluted with formic acid-ammonium formate solutions (cf. Methods). For the portion of the elution profile which is shown above, 4 N formic acid + 0.8 M ammonium formate was utilized starting with fraction 320. The *solid line* in the figure represents optical density of the eluant solution at either 260 or 290 mμ. The *dashed line* represents ribose as determined by the orcinol procedure. The fractions within the markers were pooled and utilized for the studies on uridine nucleotides.

The fractions containing the X_1 component and those containing the X_2 component were pooled separately, adsorbed on Darco G-60 and eluted as described above[d]. Since each component contained a small amount of the other uridine compound as a contaminant, the X_1 and X_2 fractions were rechromatographed separately on Dowex 1-X4 (1 cm. × 30 cm. column) using pH 5.0 formate buffer as eluent. In each case a small amount of the other uridine compound was removed by this procedure, and the X_1 and X_2 peaks thus obtained gave no indication subsequently of containing any other nucleotides as contaminants[e]. After separating the nucleotides from formate buffer using the Darco procedure, the X_1 and X_2 fractions were subjected to the studies described below.

Paper Chromatography of Uridine nucleotides. Paper chromatography of uridine nucleotides was carried out using five different solvents. In each case the unknown compounds, X_1 and X_2, were run simultaneously with commercial samples of UDP, UDPG and UDPAG. As may be seen in Table 1, R_f values from these chromatograms indicate that unknown component X_1 migrated in the same position as the commercial sample of UDPAG. Unknown component X_2 migrated in the same position as the commercial sample of UDPG in each of the solvent systems which were used.

TABLE 1

Paper chromatography of uridine diphosphate compounds

Compound	Source	R_f values				
		Solvent number				
		I	II	III	IV	V
UDP	Commercial	.17	.08	.76	.20	.20
UDPAG	Commercial	.19	.27	.67	.48	.29
UDPAG	Compound X_1	.19	.27	.68	.48	.30
UDPG	Compound X_2	.18	.22	.74	.35	.24
UDPG	Commercial	.16	.21	.74	.36	.23

[e] Peak X_2 does have a slight contamination from some non-nucleotide material washed out of the Darco G-60. This contaminant has a low absorption in the ultraviolet region of the spectrum (D_{260}/D_{280} ratio of about 1.6) with no absorption peaks. The values for uridine content shown in Fig. 2 and Table 2 have been corrected for some contamination by this material. Attempts to pre-wash the Darco G-60 to eliminate this contaminant were unsuccessful.

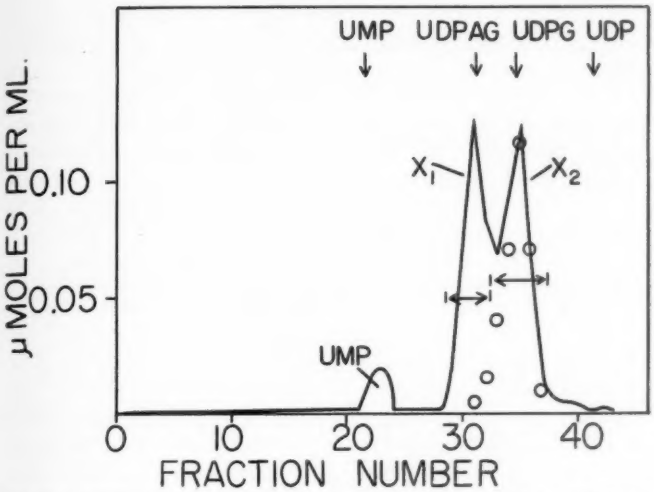

FIG. 2. Separation of the uridine nucleotides by ion-exchange chromotography. A 1 cm. x 21 cm. Dowex 1-X4 column was utilized with a pH 5.0 formate buffer as eluent as described in the text. The *solid line* represents uridine as determined from the optical density readings at 260 mμ. The *circles* represent UDPG analyses as determined enzymatically with UDPG dehydrogenase. The arrows at the top indicate the positions where the listed known components were eluted from an ion exchange column under similar conditions in a different experiment. The fractions within the markers were pooled and used for the subsequent studies described in the text.

Analyses for N-acetylglucosamine. N-acetylglucosamine determinations were carried out as described by Reissig, *et al.* (16) using commercial N-acetylglucosamine as a standard. Hydrolysis of the nucleotides to liberate N-acetylglucosamine was carried out by heating the samples for 5 minutes at 100° in 0.02 N HCl. The results of these analyses are shown in Table 2. The N-acetylglucosamine content of the X_1 nucleotide was 90 per cent of that of commercial UDPAG. N-acetylgalactosamine gives approximately 35 per cent of the color of N-acetylglucosamine by this colorimetric procedure. Consequently, the N-acetylglucosamine analysis would indicate that the X_1 component is at least 85 per cent UDPAG. However, these analyses do not eliminate the possibility that small amounts of UDP-N-acetylgalactosamine might be present in the isolated X_1 uridine compound. In order to determine very small amounts of UDP-N-acetylgalactosamine, considerably larger amounts of material would be required.

TABLE 2

Analysis of isolated uridine nucleotides for N-acetylglucosamine

Sample	Source	Uridine* μmoles/ml.	N-acetylglucosamine μmoles/ml.
UDPAG	commercial	0.075	0.078
		0.075	0.078
UDPAG	compound X_1	0.125	0.112
		0.075	0.072
UDPG	compound X_2	0.070	0.007
		0.070	0.004

* Uridine was determined from the optical density readings of the sample at 260 mμ.

Discussion

UDPG and UDPAG which were isolated from erythrocytes have been shown to have the same properties as authentic UDPG and UDPAG when subjected to anion exchange chromatography. The compounds were isolated using formic acid + ammonium formate as eluent and were rechromatographed using a pH 5.0 formate buffer as eluent. The combination of these two systems has been shown previously to be very effective in separating uridine nucleotides from other nucleotides (9). Paper chromatography using five different solvent systems showed that R_f values of the isolated compounds were the same as those of the known compounds (Table 1). The isolated UDPG has been shown to have essentially the same activity with UDPG dehydrogenase as authentic UDPG (Fig. 2). The isolated UDPAG has been analyzed for its N-acetylglucosamine content and the values obtained were within 10 per cent of values obtained with authentic UDPAG (Table 2). The above studies should satisfactorily establish the two uracil-containing nucleotides of erythrocytes as UDPG and UDPAG.

These studies indicate that the uracil-containing nucleotides are two major components which appear in the elution profile between ADP and ATP. In the particular blood specimen used in these studies, the concentration of uridine nucleotides was 5.6 μmoles per 100 ml. of cells. As seen in Fig. 2, this amount was about equally divided between UDPG and UDPAG. In several other studies, the sum of uridine nucleotides plus GDP has ranged from 5 to 7 μmoles per 100 ml. of cells. Examination of Fig. 1 would indicate that there are several minor components

in this area of the elution profile which remain to be identified. The presence of ribose (Fig. 1) in these components would suggest that they are purine nucleotide compounds. The compound with the high D_{290} values is probably GDP, which was eluted ahead of the uridine nucleotides when the large ion exchange column was used. Adenosine triphosphate ribose, which is produced by the action of acid on reduced triphosphopyridine nucleotide, might account for one of these compounds (7).

Summary

Anion exchange chromatography and paper chromatography have been utilized to separate and identify two uridine diphosphate compounds of erythrocytes. Chemical tests and enzymatic assays have been used to characterize these nucleotides as uridine diphosphate glucose and uridine diphosphate N-acetylglucosamine.

Acknowledgments

The author wishes to acknowledge the technical assistance of Mrs. Freddie Craven Hill in the performance of these experiments. He also wishes to acknowledge his indebtedness to Mr. Donald F. Fincher who collaborated in a portion of this study while he was a recipient as a medical student of a summer student fellowship. The funds for this fellowship came from a training grant to the University of Texas Medical Branch from the National Institutes of Health, United States Public Health Service.

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THE DEVELOPMENT OF A NEAR-DIPLOID *IN VITRO* STRAIN FROM A SMOKE-CONDENSATE INDUCED MOUSE TUMOR[a]

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Introduction

Ascites cells of mice and rats have proven useful as tools for the analysis of tumor development (Levan, 1956a; Makino, 1957b). Cell strains *in vitro* also have provided favorable materials because of their rapid growth and relatively easy management with the use of semi-synthetic media. In the course of *in vitro* development of most mammalian strains of both normal and malignant origin, chromosome numbers have been found to undergo changes to a heteroploid state. Such transformations are associated with structural changes in addition to the numerical increase of chromosomes (Levan, 1956; Hsu and Klatt, 1958, 1959; Levan and Biesele, 1958; Chu and Giles, 1958; Nakanishi *et al.*, 1959 a, b; Rothfels *et al.*, 1959; Nakanishi, 1960). Chromosome studies dealing with such heteroploid strains are rather difficult because heteroploid cells usually contain many small chromosomes in addition to various alterations in both their numbers and structure. Therefore, diploid strains are highly desirable for experimental work. Only a few diploid strains from normal tissue maintained after many transfers have been reported (Syverton, 1957; Tjio and Puck, 1958; Ford *et al.*, 1958; Hayflick, 1960). In the present paper chromosomal changes have been observed in the development of a near-diploid strain originated from a smoke-condensate induced mouse tumor.

Materials and Methods

Dr. Jack Neal of the Research Laboratory of Industrial Hygiene at this Medical School kindly supplied a living C_3H male mouse carrying a tumor

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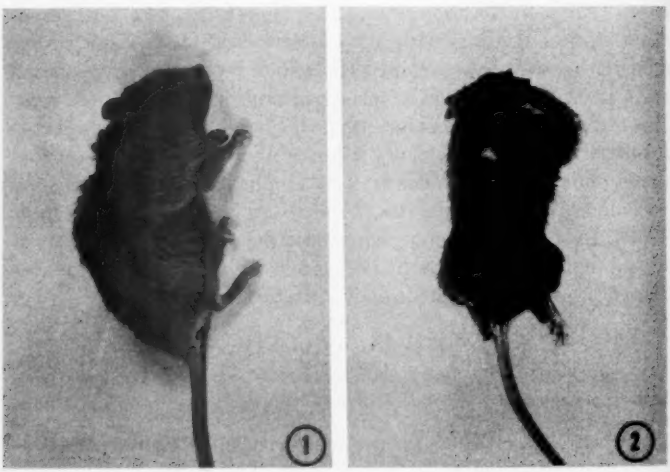
(Figs. 1, 2). This new growth was produced following subcutaneous injection of smoke condensates (Neal, 1959). Tumor tissue was cut into fragments approximately 1 mm square and cultivated in plasma clots in T60 flasks. The clot consisted of equal parts of heparinized cockerel plasma and extract from 7-day chick embryos. Four hours after setting up the cultures, 20 ml of Eagle's medium containing 10% horse serum was added to the flasks. The cultures were maintained at 37°C. The fluid medium was changed when the pH fell to approximately 6.9. On the 33rd day the 1st subculture was made from outgrowing cells. A rubber policeman was employed to scrape the cells from one-half of the floor of the flask which contained 20 ml of fresh Eagle's medium. The cells were dispersed with a 30 ml syringe fitted with a #15 gauge needle and then they were transferred to the floor of a T60 flask which was covered with a 0.4 ml plasma clot consisting of equal parts of chicken plasma and embryonic extract. The methods for further subculture were the same as those which have been described with the exception that a plasma clot was not used for the transfer of cells after the 2nd subculture. Trypsin or versene were never used to dislodge the cells. The population remaining in the original flask was treated with a colchicine solution (0.2 ml of a 1:10,000 dilution in Gey's solution plus 20 ml of Eagle's medium) for 10 hours. The method used for the spreading of chromosomes was the same as that reported by Hsu and Klatt (1958), Nakanishi *et al.* (1959b) and Mizutani *et al.* (in press). Observations on chromosomes were made with the use of phase contrast microscopes using 100X objectives combined with 8X or 12X oculars.



FIGS. 1–2. A C_3H male mouse which had a smoke condensate induced tumor.

Results

The histological study of sections of the original mouse tumor which were stained with Delafield's hematoxylin-eosin or phosphotungstic acid hematoxylin showed that the tumor here considered was a leiomyosarcoma (Fig. 3)*. According the Dr. Neal (1959), the tumors induced in C_3H mice with the tobacco condensate showed no invasion and spreading. They grew under the skin, sometimes to a size larger than the host itself without invasion, and were movable. The new neoplasms produced by him were grossly similar to the one used for the establishment of the strain line described here.

Explants showed a typical fibroblast like outgrowth (Figs. 4–7). Chromosome counts were based on reliable metaphase plates in both primary and strain cultures (Figs. 10–16). Results are summarized in Figures 8 and 9.

In the primary culture 50 metaphase figures were observed. The most frequent chromosome number was found to be 41. In 22 out of 50 cells (Figs. 12, 13, 17, 18), showed two number-dis-

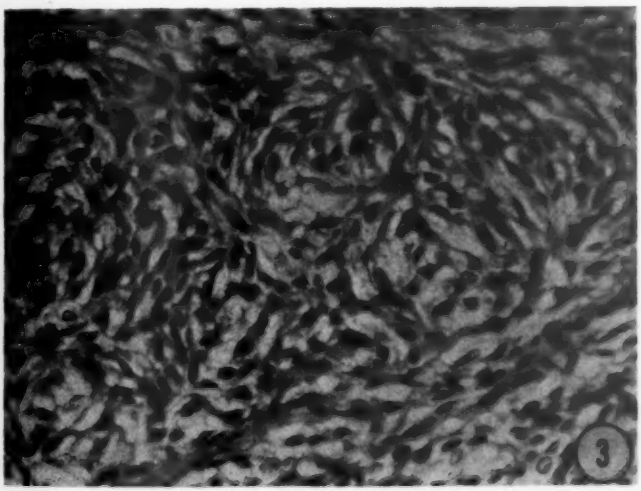
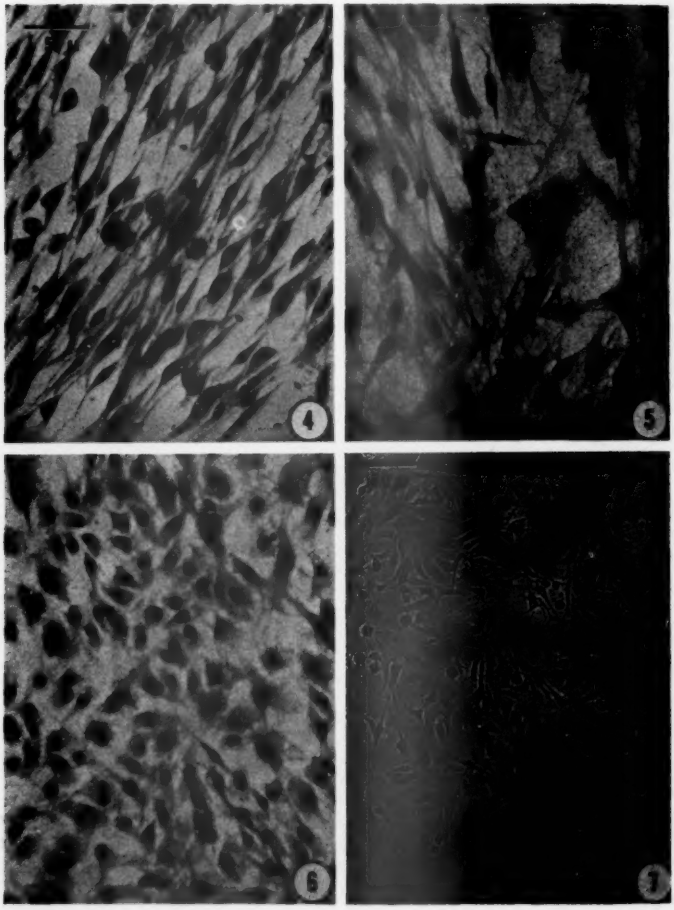


FIG. 3. Photomicrograph of the histological section of the mouse tumor.

* Courtesy of Dr. V. Stembridge.

tributions, one in the diploid range (40–43) and the other in the tetraploid range (74–82). See Figure 8, A. This fact suggests that explants contained mixed cell lines. The ratio of cells in the diploid and tetraploid ranges was about 2 to 1 (Fig. 8, A).



FIGS. 4–7. Photomicrographs showing the cell types in primary and strain cultures. FIG. 4. Fixed cellular outgrowth in primary culture, stained according to Jacobson's method. FIG. 5. Fixed cells in the 8th subculture stained according to Jacobson's method. FIG. 6. Fixed cells in the 51st subculture stained according to Jacobson's method. FIG. 7. Living cells in 61st subculture.

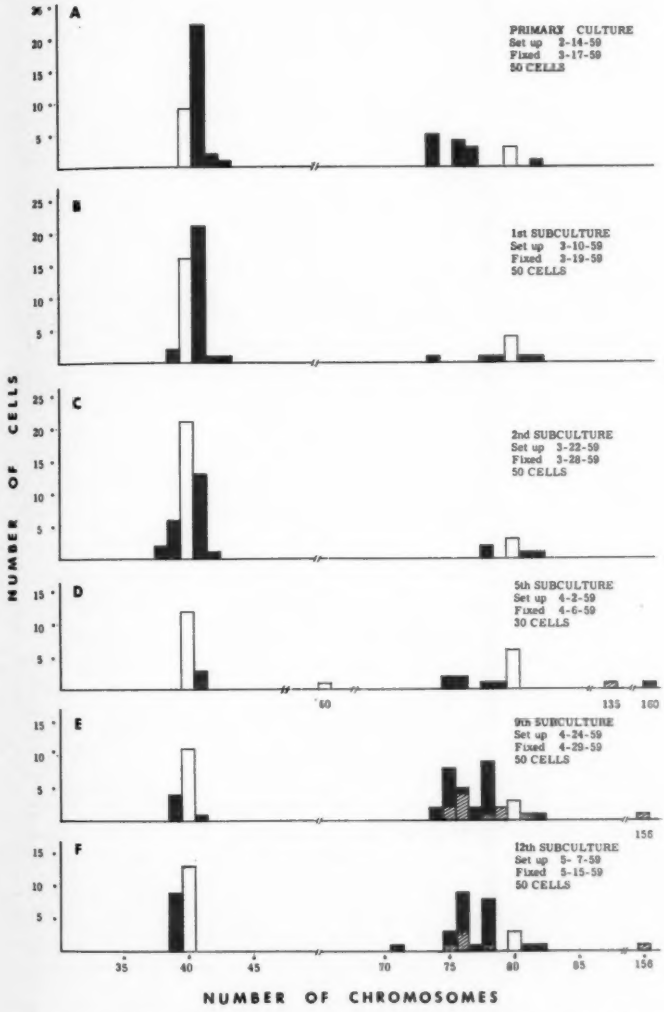


FIG. 8. Distribution of chromosome numbers in mouse tumor cells in primary and subcultures 1, 2, 5, 9 and 12.

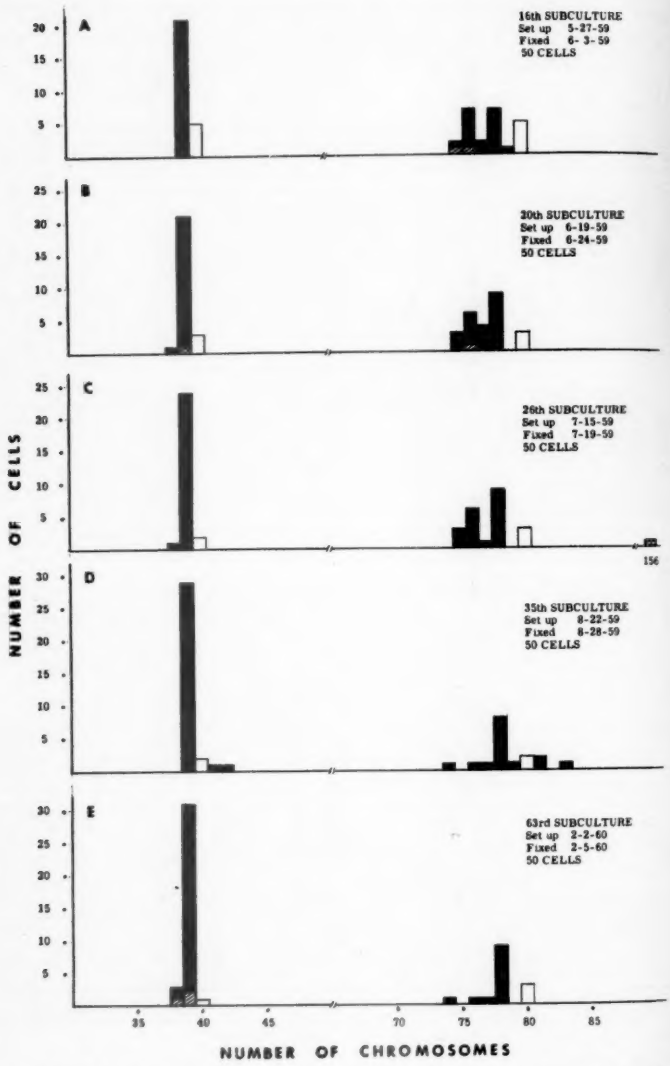
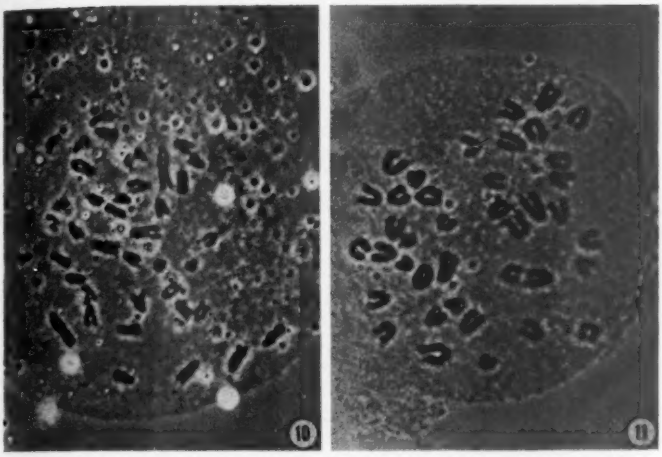


FIG. 9. Distribution of chromosome numbers in mouse tumor cells in subcultures 16, 20, 26, 35 and 63.

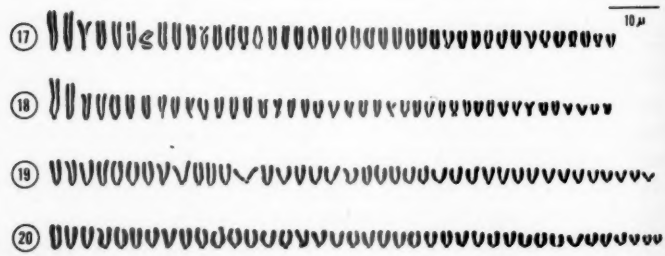


FIGS. 10–11. Photomicrographs of chromosomes in mouse tumor cells in primary and strain cultures. FIG. 10. A cell with 41 chromosomes in primary culture corresponding to the drawings shown in FIG. 13. FIG. 11. A cell with 39 chromosomes in the 26th subculture corresponding to the drawings in FIG. 15.

In the 1st subculture, cells with 41 chromosomes were dominant, since 21 cells out of 50 showed this number. The distribution of the chromosome number ranged from 39 to 42. Hypotetraploid cells found in the 1st subculture were mostly lost from view in this passage. The ratio in number of near-diploid and near-tetraploid cells was about 4 to 1 (Fig. 8, B).

In the 2nd subculture, the most frequent chromosome number was found to be 40, since 21 cells out of 50 were in this category. Cells with 41 chromosomes ranked next in occurrence, there being 13 cells out of 50. Chromosome numbers were distributed from 38 to 42 in this passage. Cells in the tetraploid range showed no remarkable change in chromosome pattern between the 1st and 2nd subcultures (Fig. 8, B-C). The ratio of cells in the near-diploid and near-tetraploid ranges was approximately 6 to 1 (Fig. 8, C). The chromosomes were again observed in the 5th subculture. Out of 30 cells, 12 had 40 chromosomes. There were fewer cells with 41 elements in this passage than in the 2nd subculture (3 out of 30 cells). The ratio of cells in the diploid and heteroploid ranges was nearly 1 to 1 (Fig. 8, D).

FIGS. 12–16. Camera lucida drawings of chromosomes of mouse tumor cells in primary and strain cultures. FIGS. 12 and 13, 41 chromosomes in primary culture. FIGS. 14 and 15, 39 chromosomes in the 20th and 26th subcultures. FIG. 16, 78 chromosomes in the 35th subculture.



FIGS. 17–20. Ideograms of mouse tumor cells in primary and strain cultures. FIGS. 17 and 18. Primary culture coresponding to the drawings in FIGS. 12 and 13, respectively. 41 chromosomes. FIG. 19. 20th subculture corresponding to the drawings in FIG. 14. 39 chromosomes. FIG. 20. 26th subculture, corresponding to the drawings in FIG. 15. 39 chromosomes.

In the 9th subculture the most frequent chromosome number was observed as 40, showing a number distribution from 39 to 41. The percentage of cells with 40 chromosomes showed a marked decrease, since 11 out of 50 were found to have that number. With the decrease of diploid cells, there was a marked increase in cells of the tetraploid range. The ratio of cells in near-diploid and near-tetraploid ranges was about 1 to 1. Chromosome numbers in the tetraploid range were distributed from 74 to 82 with two small peaks at 75 and 78. In addition to this change, metacentric chromosomes appeared in tetraploid cells and a near-octoploid cell with an incidence of 2.0% (Fig. 8, E, oblique).

Forty chromosomes were found in 13 out of 50 cells observed in the 12th subculture (Fig. 8, F). Cells with 39 chromosomes showed a remarkable increase which suggested that they might be in the process of dominating the population. Heteroploid cells showed a distribution from 71 to 82. Ten per cent of these cells contained one and, rarely, two metacentric chromosomes. The ratio of cells in the diploid and tetraploid ranges was approximately 1 to 1. The distribution of the chromosome numbers from the primary culture to the 12th subculture is shown in the histograms in Figure 8.

In the 16th subculture, cells having 39 chromosomes were dominant, since 21 out of 50 cells observed showed that number. Only 5 cells had 40 chromosomes (Fig. 9, A).

About 4% of near-tetraploid cells contained metacentric chromosomes. The ratio of cells in the diploid and tetraploid ranges was about 1 to 1.

Following the 16th subculture the modal value for the chromosomes seemed to persist at 39, with a narrow range of distribution from 38 to 42. Cells with 39 chromosomes tended to increase in number with the increase of transfer generations. As shown in Figure 9, cells with 39 chromosomes were 21, 24, 29 and 31 in number in the 20th, 26th, 35th and 63rd subcultures, respectively (Fig. 9, B-E and Figs. 14, 15, 19, 20).

The spectrum of the chromosome-number distribution in the tetraploid range was almost identical in the 20th and the 26th subcultures (Fig. 9, B-C). Metacentric chromosomes, usually one and rarely two in number, appeared in each one of the diploid, tetraploid and octaploid cells in the 26th subculture. The

ratio of cells in the diploid and tetraploid ranges was about 1 to 1 in both cultures.

In the 35th and 63rd subcultures heteroploid cells showed a peak at 78. The ratio of cells in the diploid and tetraploid ranges was approximately 2 to 1 in both subcultures.

In order to ascertain whether or not this mouse tumor strain is malignant, cells from the 9th and 10th subcultures were collected from 4 stock bottles with the aid of a rubber policeman and injected into male C_3H mice weighing an average of 20 gms. Two animals received intraperitoneal and 5, subcutaneous transfers. One of them died of an unknown cause. The mice were autopsied 3 months after injection. None showed tumor tissue. In a second series of inoculations, cells from the 44th and 45th subcultures were employed. Cells from 4 stock bottles of the tumor line were mixed with trypsinized cells which were derived from the liver, kidney, spleen and testes of a normal C_3H adult mouse. Inoculation was made subcutaneously in 4 male C_3H mice weighing about 20 gms. At the same time, the mice received an injection of cortisone acetate at a dosage of 1.3 mg per individual. No tumor was produced even after 4 months.

In the 3rd test the volume of inoculated cells was increased. Two female mice were injected subcutaneously with cells of the mouse tumor strain collected from 5 Blake bottles at the 46th and 47th subcultures. These mice also received 0.25 mg of cortisone acetate immediately after transplantation every 3 days for 3 weeks. No evidence for the development of tumor was found after 4 months. Further, in the 4th experiment three adult C_3H female mice received injections of cells from 3 Blake bottles in the 48th and 49th subcultures. Cortisone acetate was also injected at 0.25 mg per mouse. The results of observation after 3 months were still negative.

Discussion

Malignant tumors have been produced in animals through the application of carcinogenic chemicals directly to the skin or by injection into connective tissue. Many investigators have been successful in experimentally inducing cancer of skin in mice and rats with direct application of tobacco tars. Recently, Wynder *et al.* (1957 a, b) reported the development of skin carcinoma in mice through long term application of smoke condensate on

the back. Graham *et al.* (1957) induced cancers on rabbits' ears with a similar treatment. Blacklock *et al.* (1957) reported that the direct introduction of the smoke condensate into the lung of rats had produced malignant pulmonary tumors. Gellhorn (1958) found that the rate of the development of skin carcinoma in mice receiving benzpyrene plus smoke condensate was significantly greater than with either compound alone. Lasnitzki (1958) studied the effect of smoke condensates on organ cultures of human foetal lung and found that such agents increased the formation of new bronchioli and induced hyperplasia of the lining epithelium leading to partial or complete occlusion of the lumen. Nakanishi *et al.* (1959b) observed a reduction of the modal chromosome number with the appearance of dicentrics in a human foetal lung strain by treating with smoke condensate as well as benzpyrene alone and with the combination of both agents.

Histopathological diagnosis in this study revealed that a mouse tumor produced by the injection of a smoke condensate was a leiomyosarcoma.

Neoplasms have been shown to have altered chromosomal numbers and patterns *in vitro*. In the present study it was shown that the primary culture established from a mouse tumor showed 41 chromosome numbers (42%) and hypotetraploid numbers ranging from 74 to 77 (24%). Cells in short-term cultures usually showed diploid numbers and rarely tetraploid numbers. Nakanishi and Mizutani (1959) reported that chromosomes in primary cultures had the normal $2n$ number, 42 in the monkey and 52 in lamb. The normal somatic number for mice was found to be 40, all being telocentric or acrocentric. There can be arranged in a well-graded series in a descending order, and there is no marker chromosome with particular characteristics in the normal somatic complement. With ideogram analysis of the mouse tumor cells with 41 chromosomes in primary culture it was found that the first two elements were quite outstanding in size, thus being readily distinguishable from the others (Figs. 17–18). No such chromosomes occurred in normal cells. Obviously the appearance of these particular elements indicates one of the signs of chromosomal alteration occurring in this tumor. Metacentric elements occasionally found in heteroploid cells may be of a similar nature. Levan (1956b) and Ford *et al.* (1958) reported that in spontaneous and radiation induced mouse leu-

kemia and reticulosarcomas there were clear signs of chromosomal transformation with a remarkable change of chromosome number. Levan and Biesele (1958) observed such changes in a normal mouse liver in culture and reported that different cultures of the same tissue may take individual pathways for their genotypic variation.

The change in the course of the chromosome number from 41 to 39 may be explained on the following bases:

1) cells with 41 chromosomes may not have been adaptable when they were put in culture, or otherwise they failed to attach to the floor of the glass container without a plasma clot, or

2) elimination of chromosome through anaphase lagging, or nondisjunction, or

3) pre-existence of cells with 39 elements in primary culture. In addition to the numerical changes, there might have occurred many structural and mutational alterations of chromosomes in the tumor cells. It seems probable that the cells having 39 chromosomes adapted to the culture conditions and at the same time gained metabolic superiority, establishing a dominant stem line which served as a primary contributor to the growth of the tumor.

Cells containing a few micronuclei were occasionally observed in some stained preparations at the 8th subculture. This seemed to suggest the possibility of chromosomal elimination. The shift in chromosome number from 41 to 39 may have been produced by one of the mechanisms which have been considered.

The present study shows that the ratio in the number of cells in the diploid and tetraploid ranges differed considerably in earlier transfer passages, while after the 12th subculture it showed 1 to 1. Generally, cells of both normal and malignant origin, after continuous cultivation *in vitro*, showed remarkable heteroploidy with a few or no diploid cells. Hsu and Klatt (1959) established 4 sublines from the Novikoff hepatoma in ascites form which had 2 modes at 39(s) and 78(2s). Every subline showed heteroploid transformations during serial subcultures. Makino (1957a), Tonomura and Sasaki (1957) and Makino and Sasaki (1958) reported that in some rat ascites tumors the stem line chromosomes were not necessarily permanent but underwent alterations during serial transfers with changes of tumor properties. Hsu (1959) and Levan and Biesele (1958) considered

such chromosomal alterations a result of selective adaptation. The mouse tumor strain under consideration furnished no evidence for the heteroploid transformation of chromosomes as a result of observations in the course of 63 *in vitro* passages. Syverton (1957) reported in an *in vitro* strain of normal human tissue that diploid cells appeared dominant after many transfers. Tjio and Puck (1958) stated also that the chromosome number of human tissues *in vitro* remained constant at 46 for more than 5 months. In cell cultures from the Chinese hamster, Ford *et al.* (1959) found that diploid cells co-existed for a long period. Hayflick (1960) also has informed us that he had succeeded in maintaining a human strain *in vitro* which has remained at the diploid level for 10 months, from the primary to the 50th subculture.

The negative results involving transplantability of this tumor strain into mice should be re-examined with improved techniques. Chu *et al.* (1958) described that the tumor-producing capacity of the cell lines seemed to be strain specific.

Summary

A smoke condensate-induced tumor of a C_3H mouse which was diagnosed as a leiomyosarcoma was cultivated *in vitro*. Changes in chromosome numbers were observed in cells from the primary culture to the 63rd subculture. In the primary culture cells having 41 chromosomes were dominant. In passages from the 2nd to the 12th subculture, cells with 40 chromosomes were most frequent, after which those with 39 chromosomes showed a remarkable increase. Following the 16th subculture, the modal value of chromosome number was found to be 39. This value has remained without shift during passages to date (63rd subculture). Ideograms were compared for cells with 41 and 39 chromosomes.

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THE RELATION OF WATER VOLUME TO EGG PRODUCTION IN THE SNAIL *BULINUS TRUNCATUS*[a]

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Since bilharziasis is increasingly recognized as a major worldwide public health problem, there is new interest in the biology of the snail intermediate hosts of the disease. One of the foremost areas of snail study consists of questions relative to the factors involved in the production of progeny. Chernin and Michelson (1957, 1957a) found that in laboratory populations of *Biomphalaria glabratus* fecundity decreased with an increase of snail density per unit volume of water, whereas reductions of water volume on constant numbers of snails did not have a similar affect, but rather increased growth and fecundity.

Using *Bulinus truncatus* snails, the intermediate host of urinary bilharziasis in Iraq, the data of this report constitute observations on the relation of average eggs per clutch of similar size snails where different unit water volumes per snail did not vary over 30 days in the laboratory.

Materials and Methods

The *B. truncatus* snails were collected from ditches in the Alwiyah district of Baghdad, Iraq, during the first week of November, 1958, and maintained in the laboratory according to a procedure described elsewhere (Najarian, 1960). The egg production of three series of snails, each consisting of two size ranges (6.5—8.4 mm and 8.5—10 mm long), was studied. One series had snails individually isolated in small dishes having 30 ml of tap water per snail. The second series were in groups of 20 to 31 in aquaria having 100 ml of water per snail. In the third series, 10 snails were in each of the two groups with a water volume of 150 ml per snail. Egg clutches were removed daily from the containers and the number of eggs counted by means of a 10X magnifying lens or with the low power of a dissecting microscope. The water temperature during the simultaneous period of observation was 15.5—25.5°C.

Results

The egg production is summarized in Table 1. The mean number of eggs laid per clutch of snails measuring 6.5–8.4 mm long,

[a] This study was made while with the WHO Bilharziasis Control Project in Baghdad, Iraq. Paper accepted for publication May, 1960.

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TABLE 1

Egg production in the snail Bulinus truncatus *during 30 days in the laboratory*

Water volume per snail*	Snails per container	Snail length (mm)	No. clutches examined	Eggs per clutch	
				Mean	Range
30	1	6.5–8.4	156	12.5	3–21
		8.5–10	94	14.9	1–22
100	20–31	6.5–8.4	91	12.2	1–22
		8.5–10	154	13.9	2–23
150	10	6.5–8.4	110	12.8	4–20
		8.5–10	173	14.6	1–21

* These water volumes were used only because of the capacity of the containers available.

in water volumes of 30, 100, and 150 ml per snail, was 12.5, 12.2, and 12.8, respectively. With snails measuring 8.5–10 mm long, the average was 14.9, 13.9, and 14.6 eggs per clutch, respectively. Those snails which were not only individually isolated but also in a smaller water volume (30 ml) did not exhibit a decrease in average number of eggs per clutch, compared to the two series of grouped snails.

Discussion

These results indicate that, in the laboratory, variation of unit water volume per snail did not influence the average number of eggs laid per clutch of *B. truncatus*. Data reported elsewhere (Najarian, 1960a) also indicate that variations in density of *B. truncatus* per unit volume of water did not reduce fecundity, *i.e.*, eggs laid per snail per day, and in fact in some cases a denser population resulted in an increase in potential progeny. Chernin and Michelson (1957a) concluded that no one hypothesis was sufficient to explain the effects of crowding on the fecundity of *Biomphalaria glabratus*. Similarly, no causal information is known on the dynamics of *B. truncatus*. Studies with respect to the factors involved in snail fecundity must bear in mind that the production of progeny can be associated with either of two dynamics, or with their algebraic sum. One is the mean number of

eggs laid per clutch, and the other is oviposition with respect to time. When eggs per snail per day are either diminished or increased in association with water volume, snail density, or other factors, it is important to determine whether the average number of eggs per clutch, the rate of oviposition, or possibly both, are affected. The factors operative in each case could be quite different.

Summary

Studies on the egg laying of the snail, *Bulinus truncatus*, in the laboratory indicated that variations in water volume per snail had no effect on the average number of eggs laid per clutch. Snails indivually isolated also exhibited no reduction in the mean number of eggs per clutch. Any differences with respect to fecundity (eggs per snail per day) was attributed to the rate of oviposition. Laboratory investigations on snail fecundity should determine the effect of environmental factors on both the mean number of eggs per clutch and ovipositions with respect to time.

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SEROLOGY OF VISCERAL LARVA MIGRANS: *IN VITRO* LARVAL PRECIPITATE TEST[a]

LEROY J. OLSON[b]

Since the work of Sarles (1938) the *in vitro* larval precipitate test has received considerable attention both experimentally and diagnostically, for a variety of nematode infections of man and animals (see review, Thorson, 1953). In this test, the precipitates which are formed on the bodies of larvae cultured in homologous antiserum presumably represent an antigen-antibody reaction between metabolic secretions and excretions of the nematode and specific antibodies present in serum from infected hosts.

The following report presents results obtained with this test in the serology of experimental animal or suspected human infections with the dog ascarid, *Toxocara canis*, an etiological agent of visceral larva migrans (Beaver, 1956).

Materials and Methods

Stock cultures of nematodes. *Toxocara canis* eggs were obtained by dissection of uteri from gravid female worms recovered from naturally infected puppies. The eggs were pressed out mechanically or released by pepsin-digestion of uteri and incubated in 0.5% formalin at room temperature (22°C) for 40 days prior to use. Cultures of *Ascaris lumbricoides* eggs were similarly obtained from worms recovered from treated human cases on our pediatrics service.

Antisera. Each rabbit was orally given a dose of approximately 3,000 larvated eggs every 3 to 4 weeks. Blood from 6 *Ascaris*-infected, 6 *Toxocara*-infected and 6 control animals was taken by intracardiac puncture after the third feeding of eggs, and monthly thereafter as needed. In addition sera were made available by local pediatricians from children suspected of *Toxocara* infection. All sera were frozen until used.

Larvae. *Toxocara* and *Ascaris* larvae were obtained by digestion (0.75% pepsin, pH 1.5, 1 hr continuous stir, 37°C water bath) of infected mouse or rabbit tissues 7 to 10 days after stomach intubation of 10,000 larvated eggs. In more recent studies mechanical hatching of eggs has been utilized to obtain viable *Toxocara* larvae.

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In vitro test. Varying numbers of washed motile larvae (3 to 24) were added to 0.01 ml serum together with 5 micrograms of streptomycin; this preparation was placed on a cover glass which was laid over a microconcavity culture slide to make a wax-sealed hanging drop preparation. Slides were incubated at 37°C.

In anti-*Toxocara* sera, precipitates were visible on both species of larvae and in the media within 24 hrs, with a maximum reaction noted on the second and third days (Table 1). The homologous system gave the stronger reactions, although cross-reactions between *Ascaris* larvae and anti-*Toxocara* sera were apparent. Death rates of larvae (based on loss of motility and internal degeneration) increased significantly with time for *Toxocara* larvae as compared to control larvae in normal sera. Death rates for *Ascaris* larvae were similar in pattern but did not show the marked differences between experimental and control as seen for *Toxocara*.

In anti-*Ascaris* sera, cross-reactions with *Toxocara* larvae were also apparent, although as above the heterologous reaction was less apparent than the homologous reaction. Death rates of both species of larvae were not significantly greater in anti-*Ascaris* sera as compared to normal sera. The distribution of precipitates on larval bodies, while varying between protocols, tended to be

TABLE 1

In vitro ***precipitate test results with*** **Toxocara** ***(TOX) and*** **Ascaris** ***(ASC) larvae in homologous or heterologous rabbit antisera. Percentages of larvae with precipitates*** **(ppts)** ***or dead, and the amounts of precipitate in the media*** **(+)** ***are shown as averages of 4 to 6 slides/rabbit (6 larvae/slide).***

Days incubation	Anti-toxocara sera				Anti-ascaris sera				Normal sera			
	ppts		dead		ppts		dead		ppts		dead	
	TOX	ASC	TOX	ASC	TOX	ASC	TOX	ASC	TOX	ASC	TOX	ASC
1	20	15	10	15	0	20	0	40	(none)		0	0
	+	+			—	+						
2	30	15	20	35	0	10	5	70	..	..	0	10
	++	++			—	+						
3	30	2	40	65	3	10	10	100	..	..	5	80
	+++	++			+	++						
4	30	0	45	100	0	10	10	100	..	..	5	100
	+++	++			+	++						
21	..	..	100	100	..	..	85	100	..	..	60	100

most apparent at the oral and excretory zones of the nematode as shown by the data from a typical protocol in Table 2.

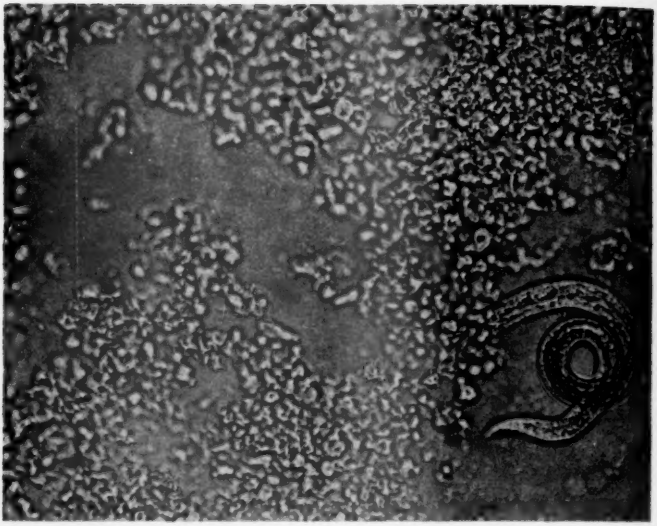
Sera from 17 children, whose histories and clinical conditions were suggestive in varying degrees of infection with *Toxocara*, have been obtained from physicians in this area within the past year for the *in vitro* test; 7 of these sera have been positive for *Toxocara*. Using this test and sera from patients with known helminth infections other than *Toxocara* the following results have been obtained with *Toxocara* larvae as a source of antigen: 2 cases of *Ascaris* (one also with *Strongyloides stercoralis*), negative; 2 cases with *Trichuris trichiura*, negative; 1 case with a recent history of *Ascaris* lumbricoides, positive. In these limited studies with human sera, no correlation has been found between *Trichinella* skin tests and serology for *Toxocara*. More clinical cases are required for testing before conclusions are indicated with respect to the specificity of *Toxocara* antigens in relation to other helminths of man. Figure 1 shows an *in vitro* precipitate reaction with *Toxocara* larvae and a positive serum from a human case; similar precipitates were noted in the rabbit system, *i.e.*, variously shaped formations which are first seen on the mouth or other body openings of the larvae, and smaller granular precipitates which are noted in the media (Fig. 2).

Studies are underway on the pattern of organ distribution for *Toxocara* larvae in mice following either primary or secondary

TABLE 2

Percent distribution of precipitates on Toxocara (*TOX*) *or* Ascaris (*ASC*) *larvae when cultured in homologous or heterologous rabbit antisera (averages of 4 to 6 slides/rabbit, 6 larvae/slide)*

Days incubation		Anti-toxocara serum				Anti-ascaris serum			
		oral	excretory pore	anal	cuticle	oral	excretory pore	anal	cuticle
1	TOX	40	30	20	10	0	0	0	0
	ASC	10	90	0	0	45	15	40	0
2	TOX	60	30	10	0	0	0	0	0
	ASC	30	40	30	0	35	30	30	5
3	TOX	50	30	5	15	100	0	0	0
	ASC	0	100	0	0	65	0	35	0
4	TOX	95	5	0	0	0	0	0	0
	ASC	0	0	0	0	90	10	0	0



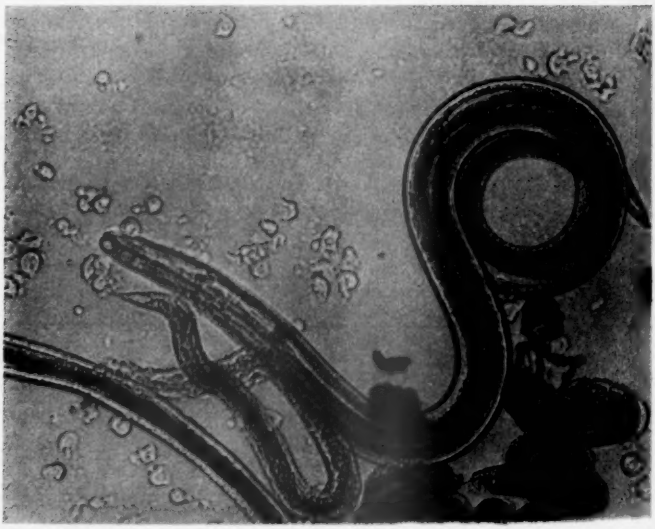


FIG. 1. *Toxocara* larvae in serum from clinical case of visceral larva migrans showing precipitates.

FIG. 2. *Ascaris* larvae in homologous rabbit antiserum showing two types of precipitates.

infection. Preliminary results with 60 mice (mixed sexes, 5 to 6 weeks of age) are shown on Table 3. Mice in group I were each given 500 larvated *Toxocara* eggs by stomach tube followed 2 weeks later by a second infection of 1000 eggs; mice in group II received only the 500 egg infection, mice in group III received only the 1000 egg infection. At necropsy 2 weeks after the time of the 1000 egg infection, larvae were recovered from these mice by pepsin-digestion of the various organs. The greatest numbers of larvae were recovered from the central nervous system and skeletal muscles, other organs had fewer numbers of viable larvae. If the differences in larval means between groups I and II are compared to their respective means in group III, it appears that some protection against the challenge infection was afforded the central nervous system of mice in group I as a result of one previous sensitization; statistical analysis of these differences showed, however, that this protection was not significant.

Discussion

The *in vitro* precipitate test was evaluated since the use of larval antigens seemed indicated in the serology of a human disease in which only the larval stage of the parasite is present. These studies show that this test, while yielding repeatable positives with known experimental and suspected clinical *Toxocara* infections, suffers from the problem noted by other workers with different tests, i.e., cross-reactions with *Ascaris lumbricoides* (Sadun *et al.*, 1957; Kagan, 1958). Kozar (1948) has also shown a cross-reaction with this test for human and pig *Ascaris* larvae.

TABLE 3

Distribution of Toxocara *larvae in mice after oral infection with larvated eggs*

Organ	Mean larval recoveries			Pct. distribution and infection period	
	Group I	Group II	Group III	2 wks	4 wks
CNS	54	12	55	26	55
Liver	11	0	7	3	0
Kidneys	12	0	1	1	0
Lung	6	0	3	2	0
Muscle	137	10	143	68	45
S. int.	0	0	0	0	0

In view of the epidemiology of *Toxocara* and *Ascaris* infections, it is possible that a child may be infected with both these nematodes; hence, the problem of serological cross-reactions becomes clinically significant. Currently we are evaluating a microculture-agar gel technique to assay the metabolic antigens of *Toxocara* and *Ascaris* in an attempt to detect common or specific antigen-antibody systems with homologous or heterologous antisera. With a test *Trichinella* system, we have noted several precipitation zones when excretions and secretions of living larvae are allowed to diffuse into the agar gel and react with similarly diffusing homologous antiserum. Metabolites from previous incubates of larvae and sonicated larval material have also been employed. It appears that at least 13 different antigens are released during a natural infection of the rabbit with *Trichinella* (Olson *et al.*, 1960). Preliminary results with *Toxocara-Ascaris* systems indicate a complexity of antigenic structure for these nematodes too. Several precipitation zones have been noted when microcultures of *Toxocara* larvae are run against rabbit or human antisera. Intra-gel absorption has been developed for this technique with a *Trichinella* test system and is now being applied to the *Toxocara-Ascaris* problem of cross-reactions. Results with this agar gel technique and *Toxocara* will be published elsewhere.

Results to date on organ distribution of *Toxocara* larvae in mice in general support the observations of Sprent (1953), and Smith and Beaver (1953) in that a high percentage of larvae invade the central nervous system of experimentally infected mice. More studies are required before conclusions may be drawn on the relationship between organ distribution of larvae and the immune state of this host. Central nervous system invasion by Ascardidae is known for a variety of nematodes and hosts (Sprent, 1955a, 1955b) including *Toxocara canis* in man (Nichols, 1956; Dent *et al.* 1956).

Summary

The *in vitro* larval precipitate test has been evaluated for use in the diagnosis of experimental and clinical *Toxocara canis* infections. Results from an experimental rabbit system show that detection of *Toxocara* antibody is possible with this test, but complicated by a cross-reaction with anti-*Ascaris lumbricoides* sera.

Similar detection was noted for sera from suspected clinical cases of *Toxocara* infection in children.

Studies on the pathogenesis of *Toxocara* infections in mice showed a high percentage of larvae had migrated, within a period of 2 to 4 weeks after infection, primarily into the central nervous system and skeletal muscles.

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BLOOD AND BONE MARROW DIFFERENTIAL COUNTS ON THE RAT[a]

R. H. RIGDON, GWENDOLYN CRASS[b] AND L. R. RICHARDSON[c]

Many observations have been made on amount of hemoglobin, percent hematocrit, number of erythrocytes and leucocytes per cubic millimeter and percentage of neutrophils, lymphocytes, monocytes, eosinophils and basophils in the peripheral blood of the rat. The average counts as given by Creskoff *et al.* (1) and Spector (5) on normal adult rats are as follows:

Creskoff *et al.*		Range	Spector		Range
Leucocytes	9.0	6–18	Leucocytes	14.0	5–25
Neutrophils	20%	2–25	Neutorphils	29%	9–34
Lymphocytes	78%	55–96	Lymphocytes	73%	65–84
Monocytes	< 1%	0–3	Monocytes	2.3%	0–5
Eosinophils	2%	0–4	Eosinophils	2.2%	0–6
Basophils	< 1%	0–0	Basophils	.5%	0–1.5

The hemoglobin is 15.6 g./100 ml. in the adult rat and 10.7 g./ml. in the newborn. The number of leucocytes also is less in the newborn (3,200 per cu. mm.) than in the adult rat (1).

We have been unable to find any differential counts on the cells in the bone marrow of the rat. Creskoff *et al.* (1) in 1949 stated that "the bone marrow of the rat is similar to that of man but is more hyperplastic. It is preponderantly erythroblastic, pronormoblastic and normoblastic. During early life the marrow is highly cellular (60–90%), becoming less cellular (30%) after the first month of life." Because of the apparent absence of data on differential count on the cells in bone marrow of adult rats, we are reporting our observations.

Methods and Materials

The rats in this study are from the Texas Agricultural Experiment Station colony in which no new strain has been introduced for at least 30 years. The

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parent generation animals in this particular study received irradiated and non-irradiated chicken and green bean diets from time of weaning. F_1 and F_2 generations were continued on these diets. The chicken and green bean diets were composed of 35% dry weight each of boned chicken and green beans and 30% of a synthetic diet with adequate vitamins supplemented. Food and water were supplied *ad libitum*. There is no indication, in our opinion, that irradiation affected either this diet or the rats that ate it. The composition of the synthetic diet has been described by Richardson and Brock (4) except that the mineral mixure was increased to 8% and 1% calcium carbonate was added. This change was made so that the final chicken and green bean diets supplied approximately 1% each of calcium and phosphorus.

The animals were kept in Wahmann type cages in an animal room where the temperature was maintained at approximately 75 degrees F during the cool months and at 80 to 85 degrees F during the warm months. Blood smears were made, fixed, and stained with Wright's stain and total leucocyte and differential counts were made by the procedures described by Hepler (3). One hundred cells were counted. Hematocrits were determined by the Van Allen (6) procedure using 1.3% sodium oxalate as the anticoagulant. Hemoglobin was determined by the cyanmethemoglobin method (2) using the cyanmethemoglobin standard.*

Differential counts were made on the bone marrow of 157 rats at time of sacrifice. These rats ranged in age from 169 to 763 days. Smears were made from the marrow of the femur and fixed immediately in methyl alcohol and subsequently stained by the Wright's technique using a Giemsa buffer. A total of 500 cells was counted on each rat. The following classification of cells was used: (1) Megaloblasts; large purple-staining nucleus with coarse chromatin. The cytoplasm is narrow and stains a dark blue-green. (2) Erythroblasts; slightly smaller (average 8 μ) than the megaloblasts. Chromatin in the nucleus is coarse and cytoplasm stains gray-blue. (3) Normoblasts; average size 7.5 μ and the nucleus is small, dense and pyknotic. Cytoplasm stains pink to slightly gray. (4) Myeloblasts; average size 10 to 12 μ with 2 to 4 nucleoli in the nucleus. Cytoplasm is narrow and blue in color. (5) Myelocytes; 8 to 10 μ in diameter and cytoplasm stains blue to pink and has a varying number of red and blue granules (all myelocytes were grouped together). (6) Neutrophils; cytoplasm is pink and contains small neutrophilic granules (includes band and segmented forms). (7) Eosinophils; the nucleus varies in shape according to age of cell. Large orange-pink granules are present in the cytoplasm. (8) Megakaryocytes; largest cell (maximum size 25 μ) with multilobulated nuclei and pink-staining granular cytoplasm. (9) Lymphocytes; large dark purple nuclei with parachromatin clumps and pale blue-staining cytoplasm. (10) Plasma cells; size 7 to 9 μ. Eccentric-placed "cartwheel" nucleus with dense chromatin. Cytoplasm stains a dense gray-blue color. The different cells are shown in Figure 1.

* Cyanmethemoglobin standard was obtained from American Hospital Supply Co.

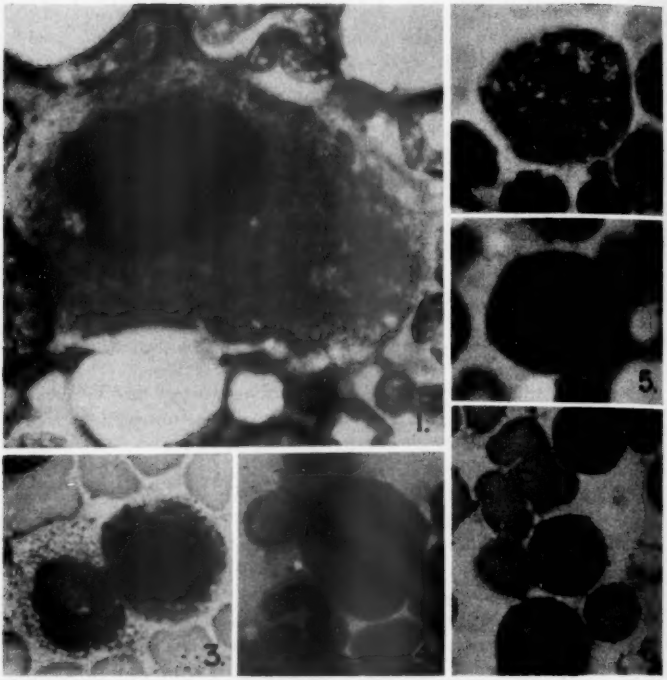

FIG. 1. Cells from bone marrow. (1) Megakaryocyte x1395; (2) Megaloblast x1710; (3) Myelocyte x1350; (4) Metamyelocyte and neutrophil x1350; (5) Plasma cell x1350; (6) Erythroblast and normoblast x1350. Wright's stain.

Results

The average cell values for the peripheral blood at various ages are given in Table 1. There is no significant difference in values for the male and female rat after the age of 90 days except that the leucocyte count is slightly less in the female than in the male. There is a lower average value for all determinations for the rats 30 days of age when compared with that of older rats. Percentage of polymorphonuclear neutrophils is higher at the youngest age. Average cell count on the peripheral blood and bone marrow of 157 rats is given in Table 2. The myeloid-erythroid ratio is also included. Values for the rat vary, as shown by the wide range in this table. Even when large numbers of cells are counted, con-

TABLE 1

Hemagram on peripheral blood of rats at various ages

Constituent	Sex	Age in days			
		30	90	254	390
Hemoglobin g./100 ml.	M	13.6	14.3	15.1	15.8
	F	15.1	14.8	15.2	15.7
Hematocrit percent	M	38.6	48.0	43.3	48.4
	F	41.6	48.5	41.0	46.6
White blood cells no.	M	10.0	16.2	12.1	11.1
$\times 10^3$/cu.mm.	F	8.8	9.3	10.5	10.6
Polymorphonuclear	M	16.2	14.3	10.7	12.5
neutrophils percent	F	14.8	16.3	13.1	12.2
Lymphocytes percent	M	79.6	82.9	86.0	84.0
	F	79.9	80.3	83.9	84.0
Eosinophils percent	M	1.3	1.3	1.3	3.2
	F	1.0	1.8	1.1	2.5
Monocytes percent	M	2.4	2.5	2.9	2.6
	F	2.7	2.4	2.4	2.6
Basophils percent	M	...	...	...	1.4
	F	...	...	...	1.2

Data for age 30, 90 and 254 days are the average of 20 male and 20 female rats. The number of animals used for data at 390 days was 52 male and 54 female rats. No basophils were identified at 30, 90 and 254 days.

siderable variation occurs not only between rats under the same conditions of diet and environment, but between several preparations from the same animal. These values, however, can be considered as representative of the rats studied.

Discussion

Cell counts on the peripheral blood in this group of rats are within the range of normal values as given by Creskoff *et al.* (1) and Spector (5). The bone marrow of the rat appears quite similar to that of man, both in the ratio of myeloid to erythroid elements and in relative distribution of cells. Morphologically the cells are almost identical. These rats showed an increase in plasma cells, eosinophils, megakaryocytes and mast cells over the usual figures for human marrow (7). This may represent the effects of infection in the rat, which is apparently a common condition among these animals.

We have no adequate explanation for the seeming discrepancy between normal rat marrow and normal rat peripheral blood.

TABLE 2

Average differential count on peripheral blood and bone marrow of 157 rats

Generation	Parent-US		^{2}F-3 US		^{2}F-3 R			
No. of rats	6	63	6	38	4	40		157
Age in days	301–450	451–763	169–300	301–450	169–300	301–450	Avg.	Range
Hemoglobin g./100 ml.	16.0	17.2	19.2	16.3	16.7	16.6	17.0	14–25
Hematocrit %	51	51	58	47	45	48	50	38–80
Leucocytes	10.0	12.4	15.0	12.9	14.0	10.8	12.5	7–22
Lymphocytes %	82	69	63	74	61	72	70	45–87
Monocytes %	1.5	1.0	1.5	0.2	2.0	0.4	1.1	0–5
Eosinophils %	1.7	0.7	1.0	1.0	1.0	1.0	1.1	0–5
Pmn. neutrophils %	14	28	35	25	35	27	27	8–57
Basophils %	0.2	0.3	0.3	0.05	0.3	0.6	0.3	0–1
Bone Marrow								
Megaloblasts	0.8	0.6	0.7	0.5	0.9	0.3	0.6	0–2
Erythroblasts	5.8	6.4	7.3	5.7	7.4	5.5	6.4	4–9
Normoblasts	34.5	25.1	23.1	18.8	21.4	18.2	23.5	12–40
Myeloblasts	0.5	0.8	0.9	0.9	0.8	1.0	0.8	0–5
Myelocytes	9.5	13.5	16.2	19.4	14.2	17.8	15.1	7–21
Neutrophils	44.0	44.2	39.9	40.6	42.8	43.5	42.5	36–49
Eosinophils	1.2	2.0	2.4	2.5	1.8	2.4	2.1	1–6
Lymphocytes	1.2	2.8	4.5	5.2	5.3	5.6	4.1	1–7
Plasma cells	1.3	2.7	2.9	4.0	3.5	3.8	3.0	1–5
Megakaryocytes	1.1	1.8	2.1	2.4	1.9	1.9	1.9	1–5
M:E Ratio	1.4:1	2.2:1	2.2:1	3.0:1	2.4:1	3.2:1	2.4:1	
M:E Range	1.2:1	1.0:1	2.0:1	2.0:1	2.0:1	1.5:1	1.0:1	
	1.7:1	4.5:1	2.3:1	4.9:1	2.8:1	4.0:1	4.9:1	

The rat marrow had a high neutrophil percentage (similar to human counts) while peripheral blood showed a relative neutropenia, averaging 27 percent neutrophils.

It should be mentioned that the mast cell was present in some but not all smears. Because it was not constant, it was not included in the differential. The mast cells are quite large (16 to 20 μ), have large purple granules, and are comparable to mast cell in human tissue. In man, the tissue mast cells appear in greater number during the healing phase of inflammation or in chronic inflammation and their histamine content is high. Whether this factor is related to the infections found in many of these animals was not ascertained. Mast cells are rare in human marrow, but conversely basophils (which are a characteristic cell of human marrow) were quite low in this group of rats.

A complete pathologic study was made on the 157 rats on which the bone marrow was studied. The majority of the animals in this experiment had a respiratory infection at time of death. A few had otitis media and some a genito-urinary infection. Although cell counts of these rats may have been affected by bacterial infections, it seems reasonable to assume that the counts made in this study are an average for the rat since the majority of all the rats that we are familiar with do seem to have a similar type of pulmonary infection. It is likely that the pathologic changes, other than the above-mentioned infection, had little, if any, effect on the hematologic values.

Summary

Cell counts on peripheral blood and bone marrow of rats are given. Some of these rats had bacterial infections involving lungs, middle ear and genito-urinary tract. These counts may be considered within the range of normal since similar infections are so frequent in the rat.

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THE CAROTID-BASILAR ANASTOMOSIS: A REPORT OF THREE CASES[a]

GLENN V. RUSSELL AND K. M. EARLE[b]

The existence of an anomalous connection of some size between the internal carotid artery and the basilar artery in man appears to have been first described by Quain (1844), and was identified as the result of the persistence of the primative trigeminal artery by Padget (1944). Until 1950 fifteen cases were reported, all found at post-mortem examination. In 1950, Sutton reported two cases identified by arteriography, one of which later came to autopsy. Since that time 15 additional cases have been reported, all diagnosed by arteriogram, and with four confirmed by post-mortem examination. Thirty-two cases of this rare anomaly have thus been reported and 20 specimens described leaving 12 cases known only from the arteriographic findings.

Three additional specimens have come to light in our Autopsy Service. When the first case was seen during the summer of 1959 we were unable to identify the large vessel which appeared to pass proximally alongside the trigeminal root, arch across the ventral aspect of the pons, and join the basilar artery. Having identified this as an example of the persistent trigeminal artery (carotid-basilar anastomosis) we were alert to this anomaly and in short order found two additional cases. Regrettably the intracranial portion of these vessels was unavailable when the brains were examined, however, there is no doubt in our minds that we have correctly identified each of these cases. During this same period the case reported by Jackson and Mercado (1960) was also found in this hospital, so that four cases have come to light in one place within a period of less than six months.

The literature on this anomaly was reviewed by Harrison and Luttrell (1953) and by Jackson and Mercado (1960), however we present in Table 1 the record of cases to date. With our three

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TABLE 1

Author	Year	No.	Origin from carotid Right	Left	P.M.	Art.
Quain	1844	1	.	1	x	
Tüngel	1860	1	1	.	x	
Duret	1874	2	2	.	x	
Tareniecki	1880	1	1	.	x	
Flesch	1882	1	.	1	x	
Hochstetter	1885	2	2	.	x	
Decker	1886	1	.	1	x	
Smith	1901	1	.	1	x	
Oertel	1922	1	1	.	x	
Hasenjäger	1937	1	1	.	x	
Sunderland	1941	.	.	.		
	1948	3	.	3	x	
Sutton	1950	2	1	1	x(1)	x
Echer	1951	1	1	.		x
Harrison & Luttrell	1953	3	2	1	x(2)	x
Sugar (cited by H&L)	1953	1	.	.		x
Montrieul & Philippides	1953	1	.	.	x	x
Stenvers, Bannenberg, & Lenshoek	1953	1	.	.	x	x
Murtagh, Stauffer, & Harley	1955	1	.	1		x
Poblette & Asenjo	1955	2	.	.		x
Schaerer	1955	1	.	1	x	x
Zaclis	1955	1	.	1		x
Mount	1957	1	1	.		x
Wise	1957	1	1	.		x
Jackson & Mercado	1960	1	.	1		x
		32	14	13	20	
Russell & Earle	1950	3	1	2	x	
		35	15	15	23	17

cases there are now a total of 35 known occurrences of the carotid-basilar anastomosis, 15 occurring on each side where this could be determined. Means of diagnosis and existence of the specimen are indicated in the columns to the right. Sunderland (1948) reported the size of his series of specimens, and other authors (see Table 2) have indicated the number of occurrences in their series of arteriograms. Taking the average it would appear that this anomaly may occur in about 0.3% of cases, Jackson and Mercado (1960) stated that it may be frequently overlooked, and suggested that the frequency of occurrence may be as much as 1% but hardly the 0.001% earlier suggested. If we have not overlooked an occurrence in some 7 years our frequency

TABLE 2

		Per cent
Sunderland	3 in 210	1.4
Sutton	1 in 1000	0.1
Harrison & L	3 in 582	0.5
Poblette & A	2 in 828	0.2
Schaerer	1 in 600	0.2
Wise	1 in 397	0.2
Jackson & M	1 in 800	0.1
Russell & Earle	3 in 640	0.5
	15 in 5057	(0.3%)

(3 in 3000 brains) would be about 0.1%, and we would suggest that a frequency of 0.1–0.3% could be expected.

Clinical findings associated with a persistent carotid-basilar anastomosis include saccular intracranial aneurysms, oculomotor palsy, and tic douloureux.

Case #1. Brain of a 30 year-old colored female with a past history of severe hypertension of 3 years duration. On her final admission BP was 250/120 and the BUN was elevated. Response to medication was poor and renal function continued to deteriorate. Patient became markedly anemic, vomited bloody fluid, and passed tarry stools. No neurological history of any significance was ever reported. Except for moderate hyperemia of the brain with an occasional RBC in the perivascular spaces, and slight arterial and arteriolar sclerosis, no significant brain pathology was noted. At the time of examination, however, an unusually large vessel was noted passing from the right trigeminal root medially to join the basilar artery. The specimen was retained and is seen in Fig. 1. The vertebral arteries, equal and of normal diameter, join to form the basilar which gives off an anterior inferior cerebellar artery on the right and internal auditory branch of some size on the left. The lower one-half of the basilar artery is somewhat reduced in size. At about its mid-point the basilar artery is joined by the carotid-basilar vessel which evidently supplied a major part of the blood that flowed rostrally into the superior cerebellar arteries and the posterior cerebrals. A posterior communicating artery of moderate size was present on the side opposite to the anastomosis, but was much reduced on the homolateral side.

Case #2. Brain of a 74 year-old white male with a long history of alcoholism and irregular food intake. On his final admission he was suffering delirium tremens, became comatose and expired. Autopsy revealed bronchopneumonia, incidental carcinoma of the prostate, moderate obesity, and traumatic absence of the right arm. The brain showed a small area of petechial hemorrhage in the left frontal area, moderate hyperemia, slight frontal atrophy, and senile plaques in the cortex. A carotid-basilar artery was noted on the left side. The specimen was retained and is seen in Fig. 2. The left

vertebral artery was markedly reduced in size. A posterior inferior cerebellar artery was seen on the right, and an anterior inferior cerebellar artery arising from the basilar on the left. The caudal one-half of the basilar was of the same diameter as the vertebral artery. At the mid-point of the basilar it was joined by a large vessel coming from the medial aspect of the trigeminal root. The basilar diameter was greater rostral to the anastomosis and gave rise to paired superior cerebellar arteries on the right and a single vessel on the left. The left posterior cerebral artery arises totally from the basilar, the posterior communicating artery on that side being a mere thread. On the side of the anomalous vessel, the first segment of the posterior cerebral artery is reduced to a thread after giving off a cluster of branches to the interpeduncular fossa. The posterior cerebral distribution is supplied from the internal carotid via a large posterior communicating artery.

Case #3. Brain of a 75 year-old white male with a clinical diagnosis of emphysema. This man had a 10 year history of chronic cough and dyspnea on exertion, of peptic ulcer, and of arteriosclerotic heart disease. He died suddenly and unexpectedly. Autopsy revealed fibrinous pericarditis. The brain showed some slight atrophy, and moderate arteriosclerosis of the cerebral vessels. At the base of the brain a large anomalous vessel joined the basilar artery from the left side. The specimen was unfortunately lost, but a sketch made at the time of examination is available (See Fig. 3). The vertebrals are equal and join to form the basilar artery which is somewhat smaller than usual. Posterior inferior cerebellar and anterior inferior cerebella arteries are present bilaterally. At the mid-point of the basilar artery a large artery joins it from the medial aspect of the trigeminal root. Rostral to this anastomosis the basilar is increased in diameter and gives off paired superior cerebellar and posterior cerebral branches. The posterior communicating artery is of moderate size on the contralateral side to the anastomosis, and absent on the homolateral side.

Except for the presence of the persistent carotid-basilar anastomosis seen in these three cases, the other variations in the vessels at the base of the brain are well described. It is to be noted that in the one instance of a reduction in the size of the vertebral (Case #2, Fig. 2) this has occurred on the side of the anomalous vessel. In one other report cited by Hasebe (1928) a markedly reduced vertebral was associated with a persistent hypoglossal artery, an anomaly similar to the persistence of the trigeminal artery, but apparently much rarer.

Reduction or absence of one or both posterior communicating arteries has been mentioned previously in association with this anomaly. In these three cases it is absent (1 time) or very markedly reduced in caliber (2 times) out of 6 chances. In two cases it is present on one side and contributes perhaps 50% of the blood which enters the posterior cerebral vessel. In the last

Fig. 1

Fig. 2

Fig. 3

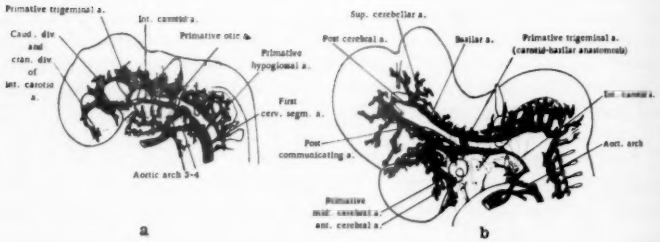

Fig. 4

FIG. 1. Circle of Willis from Case #1 viewed from below. Note the stub of the persistent primative trigeminal artery (carotid-basilar anastomosis) joining the basilar artery on the right side (left in photo). FIG. 2. Circle of Willis from Case #2 viewed from below. Note the stub of the persistent primative trigeminal artery (carotid-basilar anastomosis) joining the basilar artery on the left side (right in photo). FIG. 3. Circle of Willis from Case #3 viewed from below. Note the stub of the persistent primative trigeminal artery (carotid-basilar anastomosis) joining the basilar artery on the left side (right in drawing). (From a drawing made at the time of examination of the brain, specimen lost). FIG. 4. Lateral reconstruction of the arterial pattern in the 4 mm. (A) and the 14 mm. (B) human embryo. Note the position and relations of the primative trigeminal artery and of the posterior communicating artery. The former should be lost as the latter develops. (Redrawn from Padget, 1944).

case it is the exclusive supply of the posterior cerebral artery, the first segment of this latter vessel being the merest thread.

Considerable variation of the vessels which form the circle of Willis is well known to be the rule rather than the exception, however, the persistence of a primitive vessel, such as the trigeminal artery, is anomalous, and a rare occurrence. In the early embryo up to the 14 mm. stage (Fig. 4) the trigeminal artery is a prominent vessel supplying a major flow of blood to the mid- and hind-brain. With the appearance of the stem of the posterior communicating artery at about the 14 mm. stage, this vessel should gradually disappear. Failure of this atretic process results in the persistent carotid-basilar vessel here described, or perhaps contributes to the development of saccular aneurysms, particularly of the cavernous portion of the carotid artery. The presence of this large vessel near the IIIrd, IVth, VIth, and Vth nerves could lead to ocular palsy (reported by Murtagh, *et al.* 1955) or to facial pain (Jackson and Mercado, 1960).

Summary

Thirty-two prior reports of a persistent carotid-basilar anastomosis in man are reviewed and three new cases added and described. A frequency of from 0.1 to 0.3 percent for this anomalous persistence of the primative trigeminal artery is suggested.

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THE CHROMOSOMAL CONSTITUTION OF A HUMAN HERMAPHRODITE[a]

MOTOMICHI SASAKI and SAJIRO MAKINO[b]

It is only in recent years that human individuals carrying congenital diseases have been screened for chromosomal abnormalities, thanks to the development of simple techniques for obtaining reliable chromosome preparations from surgical or bone marrow biopsies (Ford, *et al.*, 1958; Ford, *et al.*, 1959 a, b, c; Tjio, *et al.*, 1959; Lejeune, *et al.*, 1959a, b; Chu and Giles, 1959; Hungerford, *et al.*, 1959; Jacobs and Strong, 1959; Jacobs, *et al.*, 1959; Fraccaro, *et al.*, 1959).

Available evidence presented by recent investigators, mostly employing tissue culture materials, are in general agreement that the correct chromosome number for different human races is 46, and that the X chromosome is one of the larger medium-sized submedian chromosomes, while the Y is recognized as one of the five smallest acrocentric chromosomes in the complement. Generally, the X is rather difficult to identify because of the presence of several elements which simulate its size and shape due to a continuous length variation. The Y chromosome, however, has a condensed pycnotic configuration which facilitates its identification and is therefore quite easily recognized. Without any close ideogram analysis the number of small acrocentric chromosomes, five in male cells and four in female cells, provides a useful criterion for the diagnosis of the sex of the subjects from which cells are sampled. Such a chromosomal diagnosis has made possible the identification of the genetic sex of human individuals with varying degrees of clinical hermaphroditism and some other genetic defects.

In the present study, the chromosome constitution of a case of human hermaphroditism has been investigated. To supplement clinical examinations, analysis of the chromosome complement of this individual illustrates the value of the method.

Case Report

The subject of this study was a 25-year-old Japanese diagnosed clinically as a true hermaphrodite (Fig. 1). It showed a woman-like development of the

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pelvic zone and hair growth. The breasts were uniformly soft with a slight enlargement, but no regular mammary development was observed. The patient displayed behavior typically intermediate in gesture and in general appearance, with a man-like voice. Clinical examinations revealed that the clitoris showed an unusual thickening, about 5 cm in length, with a penile configuration (Fig. 2). A rudimentary vagina was present together with a female-type urethral opening (Fig. 3). The major labia also showed unusual thickening, but no scrotal sac was present.

Through surgical examinations, it was made clear that the patient had a bipartite uterus and a small palpable body of irregular outline on the right side only, which was judged to be a gonad. Small biopsy specimens from that body examined histologically revealed a gonad containing atrophic follicles, follicular cysts and ovarian stroma. The ovarian stroma showed structures resembling testicular tissue including atrophic seminiferous tubules and interstitial tissues (Figs. 4–5). On the basis of these features the gonad was recognized as an ovotestis. A structure resembling epididymis also was observed. The germ cells showed no gametogenic activity. These histological observations made from hematoxylin-eosin preparations of formalin-fixed tissues established the diagnosis of a true hermaphrodite. No abnormality was found in the brief family history or the members so far examined.

Chromosome Studies

For the observation of the chromosomes, bone marrow biopsies were obtained from the patient by sternal puncture. The specimens were exposed to a 0.00002 percent colchicine solution for five hours. Droplets of the samples were placed on slides and an approximately equal volume of water was added to them and thoroughly mixed. After five to ten minutes, the samples were stained with acetic dahlia for five to ten minutes, and then squashed by applying an even pressure on the cover slip with the thumb.

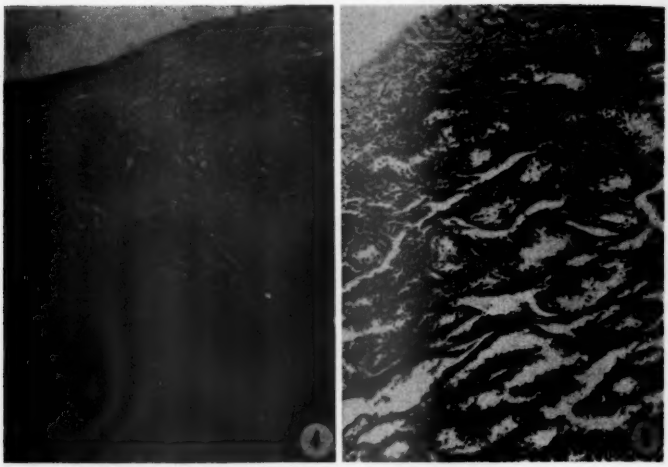
The number of chromosomes were studied in 27 metaphasic cells which permitted reliable counting. Twenty-three of the cells showed consistent counts of 46 without exception. The remaining four cells showed a deviation of the chromosome number around 46. The results are shown in Table 1. It is evident that in the majority of the cells (85.2 percent), the chromosome number was 46 (Figs. 6–10). A few elements showing a numerical variation from the normal number of 46, distributed in a

FIG. 1. General view of the patient.

FIGS. 2–3. External genitalia

1

3



FIGS. 4–5. Photomicrographs of biopsy specimens from the ovotestis. Note the ovarian stroma (4), and atrophic seminiferous tubules (5).

TABLE 1

Chromosome counts in bone marrow specimens from an hermaphrodite

Chromosome number	43	44	45	46	47	Total
Number of cells observed	1	..	2	23	1	27
%	3.7	..	7.6	85.2	3.7	100

range between 43 and 47 may have been due either to mitotic aberrations, such as nondisjunction or to technical accidents. No polyploid cells were observed.

Ideogram analyses were made of 11 cells having 46 chromosomes on the basis of their size and the position of the centromere. According to a system of nomenclature recently proposed by the Human Chromosome Study Group (Denver, Colorado, 1960), chromosomes were arranged as approximately as possible in a descending order of size. In most of the cells, however, the identification of individual chromosomes was made with great difficulty, particularly the larger medium-sized chromosomes with submedian or subterminal centromeres, since there were several chromosomes similar in size and shape, and some deformed ones,

FIGS. 6–7. Ideogram analyses of 2 cells showing female chromosome complements.

FIGS. 8–9. Two metaphase plates, camera lucida drawings.

probably due to unusual condensation as a result of the technical procedure. The arrangement of chromosomes into serial order, therefore, was quite approximate in the present study. Regardless of this difficulty, the results of the chromosome analyses in this hermaphrodite specimen were apparently identical with those from normal females. All of the cells analysed were found to have the normal female constitution with respect to the number and gross morphology of chromosomes, particularly in the finding that in every metaphase plate there were four small acrocentric chromosomes. In no cell so far analysed was any evidence for the presence of a heteromorphic X-Y pair observed. The recognition of the X chromosome was, however, a matter of considerable difficulty. In reference to recent information on the X chromosome of normal cells, two X chromosomes were tentatively designated among the submedian elements of larger medium-size, as seen in Figs. 6–7 and 10.

It is thus evident from the above findings that the genetic sex of the hermaphrodite here dealt with is female, on the basis of the chromosomal diagnosis. No observation was made with regard to sex chromatin.

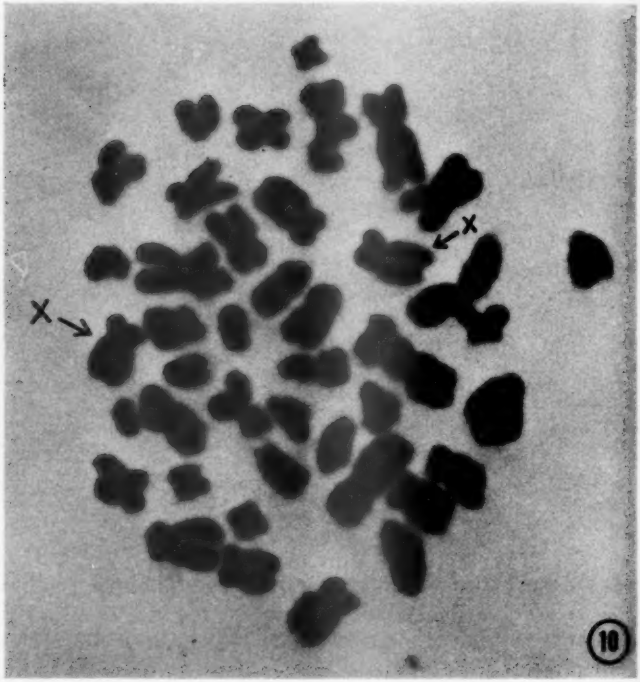


FIG. 10. Metaphase plate, photomicrograph, ca. x 3800.

Discussion

Recently, chromosome analyses have been carried out in several cases of human intersexuality which furnished subjects of special interest in the field of human genetics. Ford, *et al.* (1958) studied the chromosomes of a case of Klinefelter's syndrome, diagnosed as a female. Jacobs and Strong (1959) suggested the possibility of an XXY sex chromosomal mechanism in an apparently male-like intersex individual. Chu and Giles (1959) reported on the chromosomes of a male pseudohermaphrodite who had an XY mechanism. In cases of the Turner's syndrome, 45 chromosomes were observed and the sex chromosome mechanism interpreted as X-O (Ford, *et al.*, 1959b; Fraccaro, *et al.*, 1959). Tjio, *et al.* (1959) found 45 chromosomes

having an XO mechanism in a female patient with gonadal dysgenesis. Ford, *et al.* 1959a) dealt with a case showing both mongolism and Klinefelter's syndrome which had 48 elements and the sex chromosome constitution of an XXY mechanism. Similar evidence was also reported by Jacobs, *et al.* (1959) and Lejeune, *et al.* (1959a, b). In a case of Klinefelter's syndrome, Ford, *et al.* 1959c) demonstrated a case of the presumptive XXY-XX mosaic with an indication that the Y chromosome is strongly male-determining in man. Hungerford, *et al.* (1959) studied the chromosome pattern of a human phenotypic intersex whose external genitalia was that of a male but who had an ovotestis, and reported 46 chromosomes having an XX sex-determining mechanism. It is evident from these reports that the case presented in this paper seems to provide evidence similar to that found by Hungerford, *et al.* (1959), not only in the clinical and histopathological features of the patient but also in the chromosome constitution showing an XX sex-determining mechanism.

The most likely interpretation for the establishment of this hermaphroditic individual is the possibility of a hormonal influence probably exerted during the foetal stage. It has been shown in the bovine freemartin that the presence of a male gonad in the male member of dizygotic twins of opposite sex affects the phenotype of the female member so that it acquires male characteristics although its genetic sex remains unchanged. Presumably, the male gross appearance of the patient here considered may have been caused by testicular elements during the course of its foetal development. Male hormones produced by testicular elements might have affected the phenotype of this female and changed her into an intersex. No answer has been given for the occurrence of the testicular elements in this individual.

Summary

The patient dealt with here was diagnosed as a case of true hermaphroditism, age 25, and characterized by a penis-like thickening of the clitoris, a rudimentary vagina with a female-type urethral opening, a bipartite uterus and an ovotestis located on the right side only. The chromosomes were observed with bone marrow biopsies obtained by sternal puncture. The majority of metaphasic cells were observed to have 46 chromosomes and

the sex chromosome constitution of an XX mechanism. It is evident that, on the basis of the chromosomal diagnosis, the genetic sex of this hermaphroditic individual is female.

Acknowledgments

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This work was done at the Zoological Institute, Hokkaido University, Sapporo, Japan. The paper was prepared for publication in the Tissue Culture Laboratory, University of Texas Medical Branch, Galveston, Texas, by one of us (S. M.) with the kind cooperation of Dr. C. M. Pomerat, to whom we are grateful for his revision of the data. Thanks are also due to Mr. Roland Pomerat for his kind assistance with the manuscript.

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CYTOCHEMICAL STUDIES WITH PSITTACOSIS VIRUS BY FLUORESCENCE MICROSCOPY[a]

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Introduction

The developmental cycle of the psittacosis-lymphogranuloma group of microorganisms has received renewed attention with successive advances in cytological techniques. Bedson *et al.* (1–4), who first described the morphological changes which accompany the maturation process, used light microscopy and conventional staining procedures. Results of studies with fluorescent antibody (6, 8, 9) and with electron (13, 19), phase, and ultraviolet microscopy (18) provided additional information on the complex nature of the replication cycle. In the present study, tissue cultures infected with psittacosis agents were stained with acridine orange dye and specific cytochemical changes were observed by fluorescence microscopy. In addition, cellular changes were followed by conventional staining techniques, by the Feulgen reaction, and by immunocytochemical methods with fluorescein-tagged antibody. Treatment with ribonuclease prior to staining was incorporated into these procedures.

Methods and Materials

Tissue cultures. Human amnion (FAM) (10) and human synovial (McCoy) (15) cell lines were used. Stock cultures were maintained in the following medium A: 85 parts mixture 199 (Microbiological Associates), 10 parts lactalbumen hydrolysate (5 per cent stock solution), 5 parts heat-inactivated calf serum, 0.1 mg per ml streptomycin, and 100 units per ml penicillin. Medium A was adjusted to pH 7.2 with sterile sodium bicarbonate. Coverslip preparations were prepared in Leighton tubes (300,000 cells per ml) and incubated at 37° C. Confluent cell-sheets were evident within 3 to 4 days. Each

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cell preparation was washed twice with mixture 199 and inoculated with 0.1 ml of stock virus (100,000 LD_{50}) plus 0.9 ml of medium A without antibiotics.

Psittacosis agents. The toxigenic psittacosis agent, described by Boney *et al.* (5) from an outbreak in turkeys and characterized later by Meyer and Eddie (14), was used. This strain was maintained by repeated passage in chorioallantoic fluids of embryonated chicken eggs and will be referred to as TTS (Texas Turkey, slow strain). After rapid passage of TTS in FAM, a strain of virus evolved which was designated TTF (Texas Turkey, fast strain) (20). TTF and TTS completed their replication cycle in FAM within 2 to 3 and within 5 to 6 days, respectively. Virus titers for both TTS and TTF were determined (16) in 10 to 12 gram white mice inoculated *via* the intracerebral route.

Feulgen staining. Coverslip preparations of infected cells were fixed for 10 minutes in acid-alcohol (2 parts absolute ethanol plus 1 part glacial acetic acid) without air-drying and stained by the technique of Leuchtenberger (12).

Acridine orange staining. Coverslip preparations of infected cells were treated as follows: placed immediately into acid-alcohol for 10 minutes, rinsed in 3 changes of citrate-phosphate buffer (McIlvaine's) at pH 3.8 for 1 minute, stained with 0.01 per cent acridine orange (Harleco C.I. No. 46005, *2nd* Ed.) for 4 minutes, washed in 3 changes of buffer for 1 minute, and mounted in buffer. Reagents were made fresh each week and stored at 4° C. Stock solutions of 0.1 per cent acridine orange were prepared in distilled water and diluted 1:10 with McIlvaine's buffer prior to use.

Fluorescent antibody staining. Antiserums against meningopneumonitis virus (11) were prepared in rabbits. Fluorescein isothiocyanate (Sylvana Chem. Co.) conjugates were made with gamma globulin fractions by the procedure of Riggs *et al.* (17). All coverslip preparations were fixed in acid-alcohol for 10 minutes and rinsed rapidly in 3 successive changes of phosphate buffered saline (PBS) at pH 7.2 prior to staining by the direct (7) and the indirect procedures (21).

Ribonuclease pretreatment. Selected coverslip preparations of infected cells were treated with ribonuclease (Worthington Biochem. Corp.) prior to staining with acridine orange, Feulgen, and fluorescent antibody reagents. Preparations were fixed in acid-alcohol for 10 minutes rinsed in McIlvaine's buffer, treated with ribonuclease (40 mg per 100 ml of McIlvaine's buffer) for 1 hour at 37° C, and then stained by the above procedures. Control preparations which were incubated in the ribonuclease-carrier system (McIlvaine's buffer) alone were unaffected with respect to acridine orange color reactions. However, it should be noted that controls of fixed preparations which were incubated in PBS without ribonuclease, lost most of their cytoplasmic staining properties for acridine orange. Therefore, the use of PBS either as a carrier of ribonuclease or for prolonged washing of acid-alcohol-fixed preparations is not recommended in the acridine orange staining procedure.

Fluorescence microscopy and photography. A Reichert Zetopan microscope and illumination system was used with appropriate ultraviolet light filters. All photographs were taken with oil immersion objective (100X) and 8X oculars on Kodak, type B, Ektachrome film. Our exposure times varied from 1 to 2 minutes for acridine orange and for fluorescein-stained preparations.

Results and Discussion

Introduction. We used acridine orange in conjunction with fluorescence microscopy to visualize cytochemical changes in tissue cultures infected with psittacosis virus. In addition, host-parasite interactions were observed by immunocytochemical staining with fluorescein-tagged antibody and by cytochemical staining with Feulgen reagent. Acridine orange is a differential dye when viewed with ultraviolet light: cytoplasm and nucleoli (RNA-containing components) appear rust-red whereas nuclei and chromatin appear yellow-green (DNA-containing components). Although acridine orange does not stain DNA- or RNA-substances specifically, proof of specificity can be established by appropriate enzyme treatment. For convenience, we have referred to the yellow-green color reaction as DNA-staining and to the rust-red reaction as RNA-staining.

Nonsynchronous TTS infection. Coverslip preparations of McCoy cells were inoculated with TTS and stained with acridine orange. Observations were made throughout a 5-day period. Two to 4 hours after infection, small DNA-staining particles were seen on the cell surface and/or in the cytoplasm (Fig. 1). Size of these initial particles varied between 0.3 and 0.6 microns. They were within the size range of both the "large late and early viruses" described by Litwin (13) and the "intermediate forms" described by Tajima *et al.* (19). Between 4 to 24 hours after infection, RNA-staining inclusions appeared in the cytoplasm (Fig. 2) coincident with the disappearance of the initial DNA-staining particles. These RNA-staining inclusions first appeared as single particles (0.8 to 1.0 micron) and were later readily distinguished by their characteristic brilliant rust-red color against the lighter RNA-staining cytoplasmic background. During this period, the RNA-staining inclusions increased in size (1.0 to 4.0 microns) and in color intensity. Grape-like clusters of inclusions (2 to 20 or more individuals) were next noted, as described by Salaman (18) who used phase and ultraviolet microscopy. The appearance of these initially RNA-staining clusters could be interpreted as either 1) a multiple infection, or 2) multiplication of an initial RNA-staining inclusion, and/or 3) a manifestation of multiple growth characteristics of the virus population. During the next 48-hour period, the individual RNA-staining inclusions,

within the grape-like clusters, underwent a characteristic sequence of color changes from shades of red to orange to yellow (Fig. 3). In the following 48-hour period, DNA-staining inclusions were seen. These green-colored inclusions (Fig. 4) appeared coincident with the sequential disappearance of the red-orange-yellow-colored inclusions. Subsequently, numerous "pin-point" (0.2 to 0.4 microns) DNA-staining particles (mature virus) were noted (Fig. 4) within the green-colored inclusions. Eventually, the entire cytoplasm was filled with heterochromatic inclusions. With the subsequent appearance of intracellular and extracellular DNA-staining virus, empty vacuoles were noted (Fig. 4).

Nonsynchronous TTS infections were studied in FAM and in chicken-embryo-explant tissue cultures. Results were similar to the above mentioned TTS-McCoy system. Following cell-infection with an initial DNA-staining virus particle (as in Fig. 1), we noted the appearance of the RNA-staining inclusions (as in Fig. 2). The subsequent sequence of color changes (Figs. 5 and 6) of individual inclusions within the grape-like clusters through stages similar to those shown in Figs. 3 and 4 was noted. Throughout the 5-day development cycle, nuclei and nucleoli appeared normal. In addition, the replication cycle did not affect nuclear division, as infected cells in various stages of mitosis were seen (Fig. 5).

PLATE 1

FIGS. 1–4. Sequential cytological changes in McCoy cells following a nonsynchronous infection with TTS. Stained with acridine orange. DNA-staining cellular components are shades of green and RNA-staining cellular components are shades of red.

FIG. 1. 2 to 4 hours. Initial DNA-staning virus particle in RNA-staining cytoplasm.

FIG. 2. 24 hours. Range of size and color intensity of RNA-staining inclusions.

FIG. 3. 72 hours. Matrix of individual inclusions, within the grape-like clusters, show sequential color changes from shades of red to orange to yellow to green.

FIG. 4. 120 hours. Matured DNA-staining inclusions and pin-point virus particles. Empty vacuoles increase in numbers and reinfection occurs.

FIG. 5. 48 hours. Nonsynchronous infection of TTS in FAM cells. Initial stages are similar to those shown in FIGS. 1 and 2. Individual inclusions comprising the RNA-staining grape-like clusters, are smaller than those seen in McCoy cells. Note infected cells with mitotic figures.

FIG. 6. 72 hours. Stage following that shown in FIG. 5. Subsequent maturation of inclusion is similar to matrix-color changes shown in FIGS. 3 and 4.

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Synchronous TTS infection. Cytological changes which accompanied a synchronous infection of TTS in FAM were followed during a 5-day period. After a 30-minute adsorption interval, free virus was removed by repeated washing, nutrient medium was renewed, and preparations were examined. As described in the above section, DNA-staining particles (as in Fig. 1) were seen after 2 to 4 hours. During the following 24-hour period, RNA-staining inclusions were noted (Fig. 7) which possibly represented either a homogenous aggregate of the grape-like clusters noted during the nonsynchronous cycle of TTS in FAM (Fig. 5) or a stage in the TTF-developmental cycle described below (Fig. 13). These RNA-staining inclusions increased in size during the next 48 hour period and sequential color changes from shades of red through orange were observed (also in Fig. 7). Approximately 120 hours after cell infection, a gradual appearance of DNA-staining mature virus was noted coincident with a disappearance of the RNA-staining material (Figs. 8, 9, and 10). Repeated observations indicated that the initial DNA-staining virus had acquired an RNA-staining matrix which was contributed by the host-cytoplasm in response to viral infection. With the subsequent replication of viral material within the inclusion, the matrix was either 1) digested by the host cell, or 2) reincorporated into the cytoplasm, and/or 3) used as a metabolic pool for replication of virus. The latter possibility is suggested by the appearance of empty vacuoles and by a decrease in cytoplasmic-staining affinity for acridine orange (Fig. 10). Following the

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PLATE 2

FIGS. 7–12. Sequence of cytological changes in FAM cells following a synchronous infection with TTS. Stained with acridine orange. Initial infection (2 to 4 hours) is similar to that shown in FIG. 1.

FIG. 7. 24 hours. Appearance of homogenous, RNA-staining inclusions.

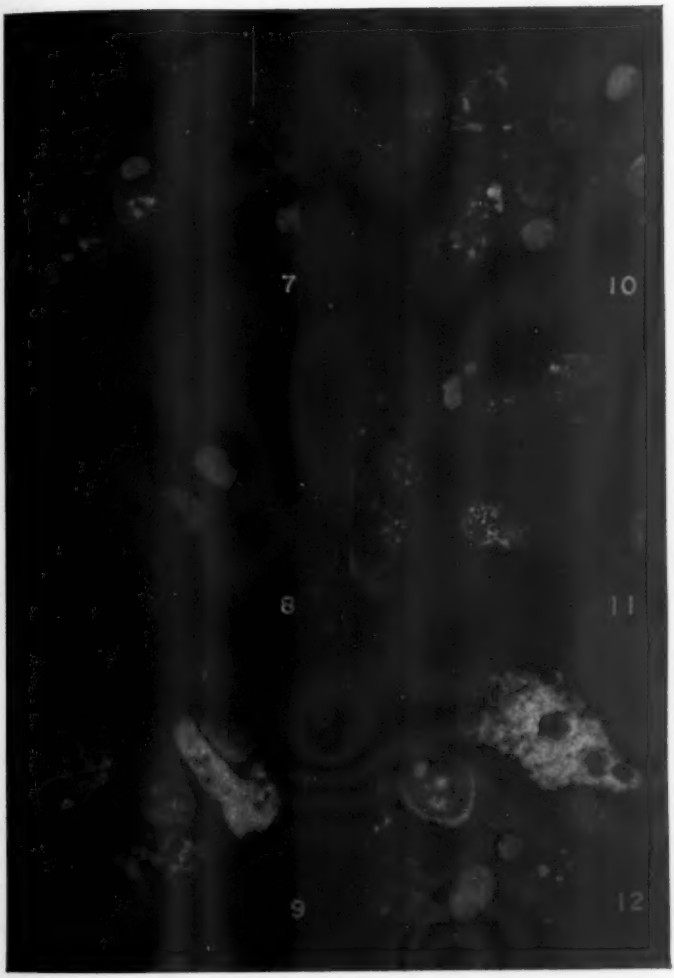
FIG. 8. 72 hours. RNA-staining matrix of the inclusions begins to disappear and DNA-staining virus particles appear.

FIG. 9. 96 hours. Disappearance of the matrix surrounding the matured virus particles becomes more evident. Massive reinfection occurs at this time and large heterochromatic inclusions are seen.

FIG. 10. 120 hours. Total disappearance of the RNA-staining matrix and subsequent decrease of the RNA-staining properties of the cytoplasm.

FIG. 11. 120 hours. Cell rupture and release of DNA-staining virus particles.

FIG. 12. 144 hours. Reinfection occurs at a nonsynchronous rate. Most stages of infection shown in FIGS 1–10 can be seen.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release of virus from the matured inclusion, a nonsynchronous-type infection was noted. Thereafter, massive heterochromatic inclusion bodies (Figs. 9 and 12) appeared and cell destruction was noted (Fig. 11).

Synchronous TTF infection. The developmental cycle of TTF in FAM differed in some aspects from the TTS-FAM cycle. With respect to time, TTF and TTS required approximately 72 and 120 hours to complete their cycle, respectively. The initial stage of infection for both virus strains started with a DNA-staining particle (as in Fig. 1). Following the appearance of the RNA-staining matrix (as in Figs. 2 and 7), certain differences were noted: 1) whereas TTF inclusions showed a gradual uniform change of matrix color from shades of red to orange (Fig. 13), and finally to green, TTS inclusions became green coincident with the disappearance of the matrix (Figs. 8, 9 and 10); 2) whereas TTF inclusions assumed a bean-shaped figure (Fig. 14) and oriented toward the region of the Goli apparatus (Figs. 15 and 16), TTS inclusions were scattered throughout the cytoplasm; and 3) whereas TTF inclusions appeared to increase in size (Figs. 17 and 18) gradually as if by a "binary fission" of individual virus particles within the inclusion, TTS inclusions appeared to "ripen" and "burst." With respect to this latter obser-

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PLATE 3

FIGS. 13–18. Sequence of cytological changes in FAM cells following a synchronous infection with TTF. Stained with acridine orange. Initial infection (2 to 4 hours) is similar to that shown in FIG. 1. In 20 hours, stages are similar to those in FIGS. 2 and 7.

FIG. 13. 24 hours. The RNA-staining matrix of the inclusion appears to "dissolve" (change from red to light orange) as the size of the inclusion increases.

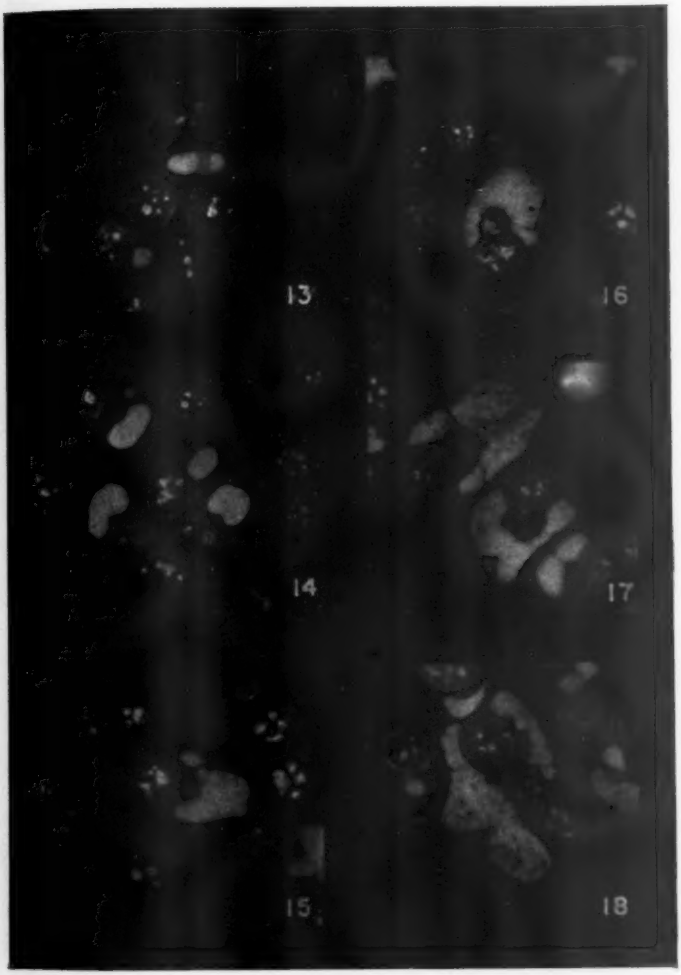
FIG. 14. 30 hours. Inclusions become kidney-shaped.

FIG. 15. 40 hours. Inclusions continue to increase in size and then orientate toward the region of the Goli apparatus which at first appears as an empty vacuole.

FIG. 16. 48 hours. Region of the Goli apparatus becomes active with RNA-staining and then DNA-staining granules conincident with a slight change in matrix color.

FIG. 17. 72 hours. Inclusions continue to increase in size with gradations in color from orange to yellow.

FIG. 18. 72 hours. Subsequently, the inclusion fills the entire cell and pinpoint DNA-staining virus particles appear. The cell ruptures as shown in FIG. 11. Throughout the entire maturation process, nuclei appear normal and mitotic figures are seen.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vation, it should be noted that the large heterochromatic inclusion of TTS (Figs. 9 and 12) resembled the later stages of TTF (Figs. 17 and 18). It is possible that the heterochromatic inclusions of TTS were formed from a massive inoculum released as a "burst" from a matured inclusion. The mechanism of this phenomenon is being investigated. The cytological differences between TTS and TTF may reflect either a dose-rate growth pattern and/or perhaps a distinction between a bacterial-type growth for TTF and phage-like "burst" for TTS. This latter possibility is suggested by the gradual sequence of matrix-color changes for TTF inclusions (Figs. 13 to 18) compared to the single-step (Figs. 8, 9, and 10) matrix-color change for TTS inclusions.

Nonsynchronous TTF infection. The nonsynchronous TTF infection in FAM was similar to the synchronous infection (Figs. 13–18). It differed from the nonsynchronous TTS-FAM or McCoy system by the absence of the multicolored grape-like clusters (Figs. 3–6). However, after repassage of TTF in embryonated chicken eggs, the TTF-FAM growth pattern reverted to the TTS-FAM growth pattern. Indeed, after serial passage of TTF in McCoy, this reversion of growth pattern was not noted. These

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PLATE 4

FIGS. 19–22. Fluorescein-tagged antibody staining of FAM cells infected with TTS.

FIG. 19. 24 hours. Stages corresponding to those shown in FIGS. 2 and 7. With or without ribonuclease pretreatment, preparations at these stages showed negative staining outlined by a pale, slightly fluorescent background which comprised the area originally occupied by the RNA-staining matrix.

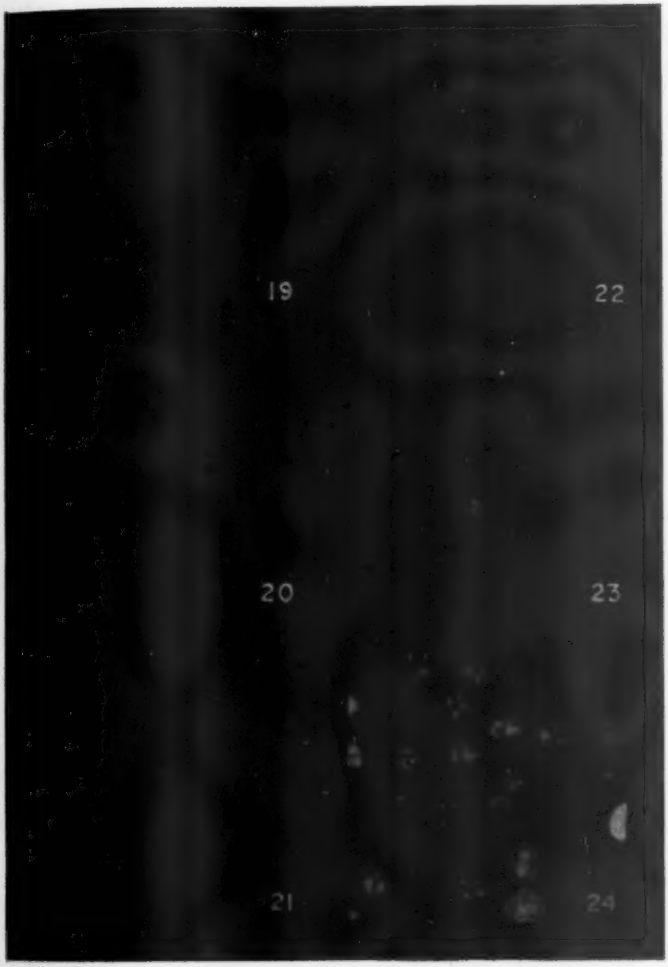
FIG. 20. 72 hours. Stage corresponding to that shown in FIG. 6 treated with ribonuclease; preparations at this stage usually showed negative staining outlined by a pale, slightly fluorescent background. Occasionally, a positive-staining inclusion was noted within the grape-like clusters (Figs. 3 and 6).

FIG. 21. 120 hours. Stages corresponding to those shown in FIG. 4 or perhaps FIG. 6 (yellow-colored matrix) were positive only with ribonuclease pretreatment.

FIG. 22. 144 hours. Stages corresponding to those shown in FIGS. 9, 10 and 12. Note the large positive-staining inclusion and the negative-staining grape-like clusters. This preparation was not pretreated with ribonuclease.

FIG. 23. 72 hours. FAM cells infected with TTS. Pretreated with ribonuclease and stained with acridine orange. Stage corresponding to that shown in FIG. 6. Only DNA-staining components remain.

FIG. 24. 144 hours. Stage corresponding to heterochromatic inclusion shown in FIG. 9. As in FIG. 23, only DNA-staining components remain.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observations reflect an intimate host-parasite relationship which affects the basic replication cycle of psittacosis virus and should be investigated in greater detail from the points of view of latency, mutation, selection, and transformation.

Effects of ribonuclease on staining procedures. Culture preparations of FAM infected with TTS were treated with ribonuclease prior to staining with acridine orange, Feulgen reagent, and fluorescein-tagged antibody. During all stages of development, the RNA-staining matrix was removed by ribonuclease and DNA-staining virus material was unmasked (Figs. 23 and 24). The viral inclusions were all Feulgen positive. As observed with fluorescein-tagged antibody, preparations characteristic of the earlier stages of virus replication (possibly up to the orange-colored inclusions within the grape-like clusters) were not antigenic (Figs. 19 and also in 20 and 22) either with or without ribonuclease pretreatment. Inclusions were outlined against a lightly stained fluorescent background which comprised the area originally occupied by the RNA-staining matrix. However, ribonuclease did unmask positive antigen from preparations which corresponded to the yellow-colored inclusions (Fig. 21). In addition, the DNA-staining green-inclusions (as in Fig. 4) and the pin-point mature virus (as in Fig. 11) were antigenic without ribonuclease treatment (Fig. 22).

The differential color specificity of acridine orange for DNA- and RNA-cellular components is indicated in part from results of experiments with ribonuclease. After treatment of infected cells with ribonuclease, only the DNA-staining (green) property of the virus and nucleoplasm (not nucleoli) remained. In other studies, it was demonstrated that the RNA-staining (red) property of acridine orange for cellular components is, indeed, a nonspecific chemical reaction. Thus, the heparin granules of mouse mast cell preparations (kindly provided by Dr. R. D. Higginbotham), which were pretreated with ribonuclease, retained their typical red color with acridine orange.

Summary

Conventional staining techniques and fluorescence microscopy with acridine orange and with fluorescein-tagged antibody were used to study the response of human amnion and human synovial

cell lines to infection with psittacosis virus. We have referred to the yellow-green color reaction of acridine orange with cell constituents as DNA-staining and to its rust-red color reaction as RNA-staining. In response to infection with psittacosis virus, the host-cytoplasm contributed a RNA-staining matrix around an initial DNA-staining particle. The development of this matrix involved a sequence of color changes from shades of red to orange to yellow to green with the subsequent emergence of mature virus. During the early stages of development, the RNA-staining matrix was removed with ribonuclease to reveal DNA-staining immature virus particles which were Feulgen positive but not antigenic to fluorescein-tagged antibody. Later stages of virus development were antigenic with the same fluorescein preparation.

The metachromatic staining properties of acridine orange fluorochrome enables visual study of cytochemical changes which accompany certain viral infections. In studies to be reported later, it was found that some antibiotics and fluorinated pyrimidines which are known nucleic acid antagonists interfered with the normal maturation process of psittacosis virus. Thus, this host-parasite, differential-staining system permits visual study of nucleic acid metabolism. In addition, this system offers a rapid and efficient means for the assay of potential chemotherapeutic agents.

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QUANTITATIVE INTERACTION BETWEEN PSITTACOSIS VIRUS AND HUMAN CELLS[a]

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Introduction

Dulbecco (2) developed a quantitative animal-virus assay technique using monolayer cultures of chick embryo connective tissue infected with Western equine encephalomyelitis virus. In this system, focal areas of cytolysis analogous to the bacteriophage "plaques" were used as a titration index. Similarly, Newcastle disease virus has been quantitated on the basis of plaque formation on monolayers of chick fibroblasts and HeLa cells and by the ability of this virus to destroy colony formation by single cells (4, 8). Since then similar techniques have been used routinely for assay of animal viruses. Weiss and Huang (10) used an "infected cell count method" for titration of feline pneumonitis virus in explanted chick embryo tissues. They describe an association between the number of inclusion bodies observed and the concentration of inoculated virus. The present paper describes quantitative growth characteristics of psittacosis virus in monolayer cultures of human cell lines in which the frequency of inclusion body formation was correlated with virus concentration in the inoculum.

Materials and Methods

Host cells. Both human amnion (FAM) (3) and human synovial (McCoy) (7) cell lines were used. Monolayer cultures of these cell lines were prepared on coverslips in Leighton tubes containing 1.0 ml of nutrient medium: 85 parts mixture 199, 10 parts lactalbumen hydrolysate (5 per cent stock solution), 5 parts heat-inactivated calf serum, and 0.1 mg per ml streptomycin. This medium was adjusted to pH 7.2 with sterile sodium bicarbonate.

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Virus strains. The toxigenic psittacosis agent, described by Boney *et al.* (1) from an outbreak in turkeys and characterized later by Meyer and Eddie (6), was used. This strain was propagated in chorioallantoic fluids of embryonated chicken eggs and was designated TTS (Texas turkey, slow strain). After rapid passage of TTS in tissue cultures of FAM cells, a strain of virus evolved which was designated TTF (Texas turkey, fast strain). Virus stocks of the 16th FAM passage of TTF and the 18th McCoy passage of TTF are described in this paper. Virus titers (LD_{50}) were obtained by assay in 10 to 12 gram white mice inoculated (0.03 ml) *via* the intracerebral route (9). As TTF caused detachment of infected cells from glass, this phenomenon was also used as a means of determining virus titers in tissue cultures ($TCID_{50}$). Cell sheets contained in stationary tubes were each inoculated with 0.3 ml of 10-fold dilutions of virus. The tubes were supplemented with 0.7 ml of nutrient medium. Titers were determined after 8 days incubation at 37° C.

Infected cell count titration. TTF stock virus (0.3 ml) was inoculated onto sheets of FAM and McCoy cells which had been washed previously with fresh nutrient medium. After a 30 minute adsorption period at 37° C, 1.5 ml of nutrient medium was added to each tube and the cultures were reincubated. At time intervals, coverslip preparations were stained with Wright's plus Giemsa and cytoplasmic inclusion bodies were scored using a 600 X binocular microscope. The average number of FAM and McCoy cells per field was 40± 7.1 and 60±9.3, respectively. The initial TTF inclusions in McCoy cells became visible in the cytoplasm 12 hours after inoculation. The percentage of infected cells reached a maximum within 24 hours, after which time secondary infections occurred. As reinfection limited the accuracy of this method, all cultures were examined 24 hours after inoculation.

In this method, it was assumed that one virus particle is sufficient to infect one cell and that the number of infected cells is thus proportional to the number of virus particles. These assumptions are valid provided the percentage of infected cells is small (10). Multiple infections per cell were noted when the number of infected cells exceeded 20 per cent of the total number of the available cell population.

Results

Frequency distribution of infected cells. The assumption that there was a random distribution of virus particles on coverslip preparations was tested. It is reasonable to expect that the virus distribution will follow the Poisson distribution if the uniformity of cells and coverslip surface provided equal opportunity for each virus particle to infect.

Four day McCoy cell preparations were inoculated with a dose of TTF estimated to give a minimum ratio of 1 virus particle to 10 or more cells. After incubation of cultures for 24 hours, the number of infected cells with inclusions was counted in a total of 100 to 130 fields per coverslip.

As shown in Table 1, the observed frequency distribution of infected McCoy cells was equivalent to the theoretical distribution. Three typical experiments are listed in which the average number of infected cells per microscope field (M) ranged from 1.2 to 2.5. With other M values in the range of 0.4 to 4.0, a random distribution of virus inclusions was also noted.

Multiplicity of infection per cell. The distribution of virus particles among individual cells also follows the Poisson formula provided that all cells are considered equally susceptible to infection. The Poisson distribution is expressed as follows:

$$P_x = \frac{m^x}{x!} \cdot e^{-m} \qquad (1)$$

where P_x is the probability of any cell becoming infected with x virus particles when the average multiplicity of infection per cell is m. If it is assumed that a single particle can infect a single cell, then the number of surviving cells (uninfected) is given by the first term of the Poisson series:

$$e^{-m} = \text{fraction of surviving cells or} \qquad (2)$$

$$m = -\ln (\text{fraction of surviving cells}) \qquad (2')$$

Thus, the logarithm of the fraction of surviving cells is inversely proportional to the multiplicity of infection (m). In addition, the

TABLE 1

Frequency distribution of McCoy cells with inclusion bodies

No. of cells with inclusions per microscopic field (100 X) (x)	Tube no. 1 M=2.5‡ Theor.*	Obs.	Tube no. 3 M=2.0 (x)	Theor.	Obs.	Tube no. 5 M=1.2 (x)	Theor.	Obs.
0	10.9	12	0	13.5	15	0	30.1	30
1	27.4	27	1	27.1	26	1	36.2	39
2	34.4	34	2	27.1	24	2	21.6	21
3	28.5	32	3	18.0	18	3	8.7 | 12.1	7 | 10
4	20.6	14	4	9.0 | 14.1	11 | 17	>4	3.4	3
5	7.9 | 11.9	10 | 15	5	5.1	6		$\chi^2 = .702$	
6	2.8	4		$\chi^2 = .974$			$.20 < P < .30$	
7	.87	1		$.10 < P < .20$				
>8	.33	0						
	$\chi^2 = 3.02$							
	$.30 < P < .40$							

* Calculated by Poisson's formula.
‡ M means average number of infected cells per microscopic field.

value m should be proportional to the concentration of input virus. This is represented by a linear relation between the logarithm of the fraction of surviving cells and the concentration of input virus. If, however, two particles are necessary for establishment of a cell infection, then the probability of cell survival is:

$$P_0 + P_1 = e^{-m} (1 + m) \quad (3)$$

and if three particles are necessary (5):

$$P_0 + P_1 + P_2 = e^{-m} (1 + m + \frac{m^2}{2}). \quad (4)$$

Coverslip preparations of both FAM and McCoy cells were inoculated with dilutions of TTF and multiplicity of infection per *individual* cell was studied. The relationship between virus concentration and multiplicity of infection per cell was calculated from the proportion of uninfected (surviving) cells based upon their Poisson distribution. Results of two such typical experiments are summarized in Table 2. Using formulas 2 and 2′,

TABLE 2

Relationship between virus concentration added and multiplicity of infection calculated from proportion of uninfected cells based upon the Poisson distribution

Exp. no.	Virus stock used	Dil. of virus	Host cell used for assay	No. cells counted	Fraction of surviving cells, measured by absence of inclusions	Multiplicity of infection, m.*	Relative value Obtained	Ta
1.	TTF–16† (FAM)	Undil.	FAM	393	.0152	4.37	16.0	1
		1:2		129	.0852	2.50	9.2	
		1:4		461	.226	1.56	5.7	
		1:16		907	.753	.274	1.0	
		1:16	McCoy	317	.0915	2.43	8.9	
3.	TTF–18 (McCoy)	Undil.	FAM	391	.315	1.19	12.7	1
		1:2		544	.467	.77	8.3	
		1:4		940	.723	.32	3.5	
		1:8		798	.816	.20	2.2	
		1:16		925	.914	.093	1.0	
		Undil.	McCoy	1,458	.0014	6.84	31.1	3
		1:2		972	.0268	3.67	16.1	1
		1:4		296	.132	2.02	9.3	
		1:8		385	.389	.95	4.3	
		1:16		1,196	.635	.45	2.05	
		1:32		668	.801	.22	1.0	

* Multiplicity of infection, m, is given by the following formula: m = −ln (No. cells without inclusions/No. total counted).

† TTF–16 (FAM) means a TTF virus stock of the 16th passage in FAM cell.

‡ Calculated from reciprocal of virus dilution.

the multiplicity of infection per cell was shown to be proportional to the concentration of input virus over a fairly wide range. Figure 1 is a comparison of data in Table 2 with the theoretical curves expressed by formulas 3 and 4. These results support the

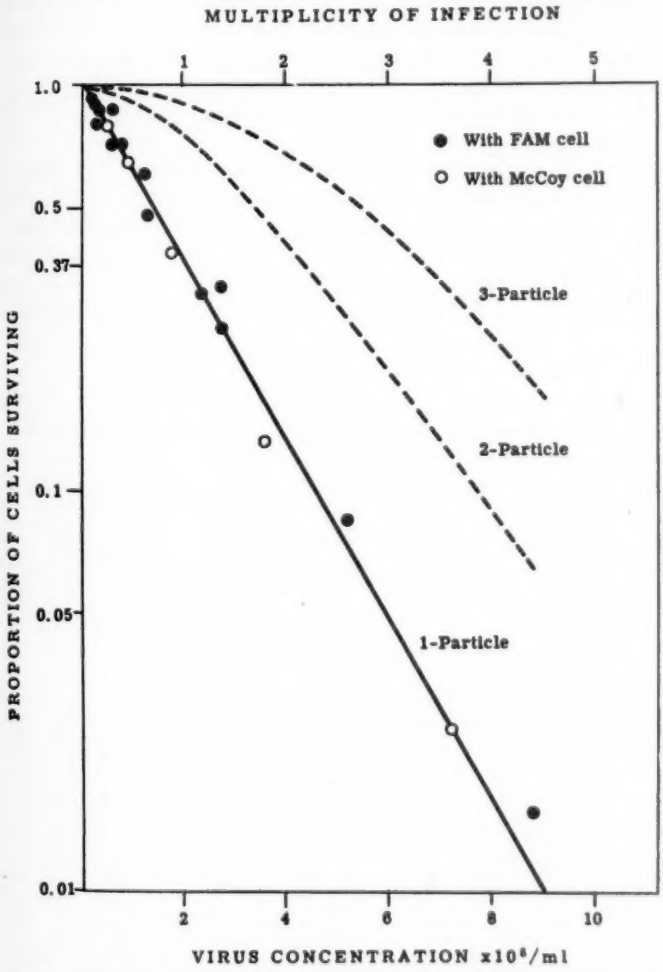

FIG. 1. Logarithmic relation between virus concentration and multiplicity of infection per cell as calculated from the proportion of surviving cells.

following conclusions: 1) Poisson's formula is applicable for analysis of the quantitative interaction between TTF and susceptible cells, 2) each TTF virus particle has the potential to infect a susceptible cell by itself, 3) multiplicity of infection is a useful index for the titration of infective units, 4) since more than 99 percent of cells exposed to a heavy virus inoculum formed inclusion bodies, every cell is considered uniformly susceptible to infection, and 5) as will be demonstrated in the following section, McCoy cells were more susceptible to infection than were FAM cells.

Comparison of sensitivity of cell lines to infection. The susceptibility of both FAM and McCoy cells to infection with the two TTF stocks is shown in Table 3. It is evident that McCoy cells are more susceptible to infection than FAM cells. A more direct demonstration of the susceptibility gradient of McCoy cells to infection is seen in Table 4. The number of TTF-infected McCoy

TABLE 3

Comparison of infective units assayed on both FAM and McCoy cells by the infected cell count method

Exp. no.	Virus stock used	Dil. of virus	Host used for assay	Infective units of original stock $X10^6$/ml.		Ratio Inf. Units on McCoy / Inf. Units on FAM
				For each dilution	Mean ± S.D. of mean	
1.	TTF-16 (FAM)	Undil.	FAM	5.9	6.7 ± .75	11.5
		1:2		6.6		
		1:4		8.3		
		1:16		5.9		
		1:16	McCoy	76.9		
3.	TTF-18 (McCoy)	Undil.	FAM	1.6	2.1 ± .54	6.9
		1:2		2.0		
		1:4		1.7		
		1:8		2.1		
		1:16		3.0		
		Undil.	McCoy	13.7	14.4 ± 1.1	
		1:2		14.7		
		1:4		16.2		
		1:8		13.2		
		1:16		14.4		
		1:32		14.1		

TABLE 4

Comparison between numbers of infected cells per unit area of monolayer cell sheet of two different host cell lines exposed to a same dosage of virus inoculum

Virus stock	Added virus dilution	Average number of infected cells per microscopic field with standard deviation of mean: McCoy (a)	FAM (b)	Ratio a/b
TTF–18 (McCoy)	1:10	29.4 ± 2.5 (10)*	3.4 ± 1.0 (40)	8.7
	1:100	3.4 ± 1.3 (50)	.37 ± .33 (60)	9.2

* No. of fields examined.

TABLE 5

Comparison between the infective units of TTF virus assayed by tissue culture methods and LD_{50} of mouse intracerebral (i.c.) inoculation

Exp. no.	Virus stock used	Host cell used for assay	Titer of units determined by the infected cell count method/ml. (a)	Reciprocal of $TCID_{50}$ endpoint of cytopathogenic changes in tubes per ml.	Reciprocal of LD_{50} mouse i.c. inoculation/ml. (b)	Ratio a/b
1a.	TTF–16 (FAM)	FAM	$10^{6.82}$	$10^{7.02}$	$10^{7.52}$	0.20
2.	TTF–16 (FAM)	FAM	$10^{5.86}$	N.T.*	$10^{6.52}$	0.22
3a.	TTF–18 (McCoy)	FAM	$10^{6.22}$	N.T.	$10^{7.19}$	0.11
1b.	TTF–16 (FAM)	McCoy	$10^{7.89}$	$10^{7.52}$	$10^{7.52}$	2.4
3b.	TTF–18 (McCoy)	McCoy	$10^{7.14}$	$10^{7.42}$	$10^{7.19}$	0.99

* Not tested.

cells per unit area of cell sheet is approximately nine times that found in FAM cells which had been inoculated with the same dose of virus.

Comparison of titers by mouse inoculation, by tissue culture assays, and by infected cell count method. As shown in Table 5, the titers obtained by the infected cell count method using McCoy cells are almost equal to the mouse inoculation titers whereas the former technique using FAM cells is less sensitive. It should be noted, also, that the $TCID_{50}$ determinations with McCoy cells, are approximately similar to those titers obtained by the infected cell count method.

Summary

Quantitative interaction between an enhanced psittacosis virus, strain TTF, and its host cell grown on coverslips was studied by means of the "infected cell count method" described by Weiss and Huang (1954).

Fraction of cells surviving exposure to various amounts of virus agreed with theoretical values calculated according to Poisson's formula, over a fairly wide range. Multiplicity of infection is considered as a useful index for the titration of infective units of the virus. The above evidence supports a hypothesis that each TTF virus particle has the potential to infect a susceptible cell by itself. Since more than 99 per cent of cells exposed to a heavy virus inoculum formed inclusion bodies, every cell is considered uniformly susceptible to the virus infection.

It was shown that the McCoy cell line was extraordinarily sensitive towards the TTF virus infection: as sensitive as the inoculation of mice by the intracerebral route.

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MUCOPOLYSACCHARIDES OF NORMAL AND TUMORAL FIBROBLASTS CULTURED *IN VITRO*

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The isolation of hyaluronic acid and its corresponding enzyme, which is so important as a diffusion factor, opened a new era in the investigation of connective tissue.

The composition and nature of ground substances of connective tissue have been established by chemical methods, but it was necessary to know more about the origin of the isolated substances and the role performed by fibroblasts in the formation of connective tissue matrices. The tissue culture method was employed. Vaubel (19) first demonstrated the presence of polysaccharides "in vitro" by production of mucine clot in liquid culture of rabbit synovial cells. Later Grossfeld (6), Kling (12), Castor (2), Hedberg (10), confirmed Vaubel's results in cultures of normal and pathological human synovial cells. They determined quantitatively, by viscosimetric, turbidimetric and chemical methods, the types of mucopolysaccharides and the products of their degradation in the culture medium.

Grossfeld *et al.* (7,8,9) and Berenson (1) in a series of experiments on cultures, determined the nature of the substances produced by synovial cells and also by other types of embryonic tissues of mesodermal origin. It was then possible to classify the types of fibroblasts of different origin, according to the type of mucopolysaccharides produced "in vitro."

The composition of polysaccharides in extracts of tumors of mesenchimal origin have been studied since 1939; by Kabat (11) in two chicken sarcomas and by Pirie (14) in the Rous sarcoma and in the Fuginammi mixo-sarcoma. Pirie extracted a substance similar to the hyaluronic acid, isolated from the human umbilical cord.

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In an extract of Rous sarcoma Warren (20) observed a type of mucopolysaccharide less viscous than umbilical hyaluronic acid and Sylven (17) extracted a sulphuric ester of a complex mucopolysaccharide, similar to heparin from the fundamental substance of mesenchymatous tumors.

Sylven (18) found alterations in the connective tissues surrounding certain tumors. He noticed the presence of an amorphous and metachromatic substance, among the collagenous fibers that was totally digested by testicular hyaluronidase (Cinti and Mignani) (3). Moreover, Mancini *et al.* (13) observed in the peritumorous connective tissue, a great formation of mucopolysaccharide acid, digestible by hyaluronidase and Eguia (5) by means of histochemical reactions, found mucopolysaccharide acids (hyaluronic acid type and chondroitin sulphurous type), in the amorphous substance of the stroma surrounding the tumor.

The production of mucopolysaccharides has not yet been studied in tumor cells cultured "*in vitro*" to clear up the problem of the origin and biological significance of these substances in relation to cellular proliferation and regeneration of tissues. In the present study by means of cell culture we have undertaken the task of ascertaining whether sarcoma fibroblasts fidder from normal ones in their capacity to synthesize mucopolysaccharides.

Materials and Methods

Small fragments of (1 x 1.5 mm) were prepared from:

a) Sarcoma 180, maintained in the C_3 H/b.a. mouse strain.

b. Peritumoral connective tissue.

c) Connective tissue from a zone distant from the tumor.

These fragments were washed in balanced salt solution and cultivated on the walls of tubes with a thin layer of chicken plasm to give adherence to the glass.

As cultures of sarcoma 180 in plasma clot rapidly degenerate they were also trypsinized in order to permit long term cultures. The nutritive medium consisted of Hanks Sims 70% and human serum 30%. Adjusted pH to 7.6 and cultured at 37 C.

Estimation of Hyaluronic Acid

From the beginning of the cellular migration samples of culture medium were taken every 48 hours in order to determine the amount of mucopolysaccharides.

As a control we used nutritive medium incubated without explants. The turbidometric method was used for quantitative estimation of mucopolysaccharides in the culture medium.

Since the turbidity produced when hyaluronic acid is mixed with acidified

protein is a function of the concentration of the polysaccharides, Seastone and Kass (15) reference turbidity curves may be plotted by adding known amounts of purified hyaluronic acid to acidified protein under standard conditions. To obtain the curve of turbidity we used purified hyaluronic acid isolated from umbilical cord (Chemical Company) at concentrations of 20 to 200/ml in phosphate buffer (pH 6). The acidified protein solution was prepared by diluting horse serum 1:10 with 0.5 acetate buffer (pH 4.2) and adding 4N ClH until pH 3.1 is reached.

In all our experiments the standard method used for turbidimetric measurements consisted in adding the polysaccharide (usually contained in a volume not exceeding 1 ml) to 0.5 acetate buffer (pH 4.2) to bring the final volume to 4 ml. To this solution 1 ml of the acidified serum was added; the turbidity was allowed to develop for 30 minutes and determined with a photo-electric colorimeter using a 530 mμ filter. The curve is shown in Fig. 1.

To test the medium by previous hydrolysis with hyaluronidase a standard amount of polysaccharide was incubated with variable enzyme solutions. At

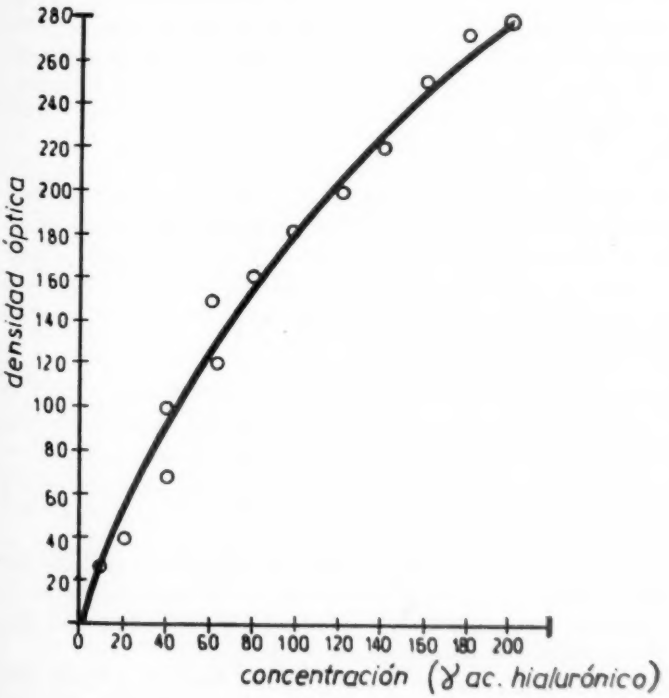

Figure 1

regular intervals, samples of the reacting mixture were removed. After 30 minutes at room temperature the turbidity was read as previously outlined. In our experiments we found that 50 U (T R U) Unidasa (hyaluronidase in phosphate buffer) was the optimum necessary to reduce turbidity to 50%.

For the histochemical study of cultures and explants were prepared on cover glasses placed in tubes and fixed in Carnoy's and Bovin's and stained directly with 1% toluidine blue to study metachromatic reaction at pH 4–3, while other explants were previously included in paraffin for the study of microscopic details. The removal of metachromatic reaction was obtained by incubation with hyaluronidase.

Results

Growth and cellular morphology.

a) Culture of tumor cells from sarcoma 180. The sarcoma cells which begin to migrate sooner than normal cells degenerate quickly. Only trypsinized cells could be maintained by subculturing during a long time. The study of tumor cells with the metachromatic reaction did not reveal the presence of intracytoplasmatic or extracellular granules. Only in cases of mixed explants of tumoral and peritumoral tissue could extracellular granules be observed.

b) Culture of normal connective adult peritumoral tissue. This tissue takes generally 48 hours to show outgrowth. Characteristic fibroblasts are observed migrating in large number and in a reticular pattern. After the third day of growth "in vitro" a large number of cells with abundant cytoplasmatic granules appeared which lasted for 15 days. The granules became extracellular on the 5th day.

Estimation of mucopolysaccharides in culture medium.

We determine mucopolysaccharide in the fluid samples extracted from the following culture media: incubated, from sarcoma 180, from peritoneal tissues, from connective tissue distant from the tumor. These cover 48 hour intervals between the 2nd and 30th day.

Turbidimetric curves of culture medium from sarcoma 180 showed that the amount of mucopolysaccharides found in all cases was very small with a slight increase on the 16th and 18th day and decreasing from then on. (Fig. 2)

Turbidimetric curves of culture fluids from peritumoral connective tissue showed that from the 3rd day mucopolysaccharides

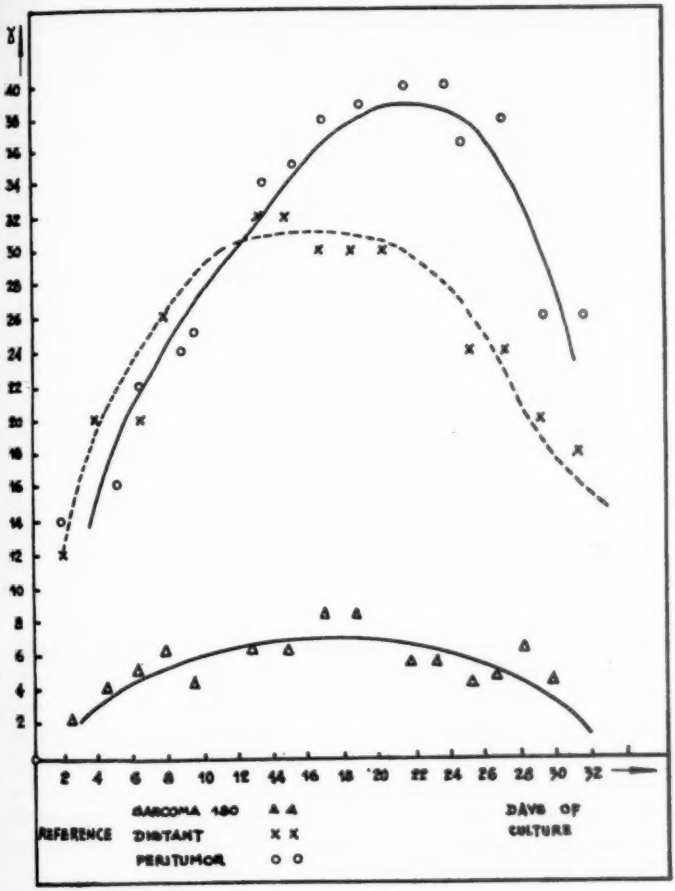

FIGURE 2

in culture medium increased gradually and reached its highest value between the 16th and the 26th day, then decreased. (Fig. 2)

Turbidimetric curves of culture fluids from normal fibroblasts of distant connective tissue showed that from the 2nd day mucopolysaccharides in culture medium increased gradually and

reaching their highest value between the 12th and the 14th day, then decreased. (Fig. 2)

All determinations made on material previous hydrolysed by the enzyme save negative turbidity. The mixed cultures of sarcoma and peritumoral tissues showed amount of mucopolysaccharides higher than those observed in the cultures of pure sarcoma probably because sarcoma cells take longer to degenerate.

Analysis of the variance (Snedecor 16) was used to determine the significance of the values obtained in each zone studied by cell culture. The differences between the data from each zone are statistically significant. (Fig. 3)

FIGURE 3

Analysis of variance to determine significance of the values in the different zones

Source of variation	Degree of freedom	Sum of squares	Mean square	F
Group	2	15,226	7,613	113
Individuals	42	2,844	67	

$n_1=2$.
$n_2=42$.
$F=5.15$ for $P=0.01$.

Discussion and Summary

We have confirmed the results of other authors referring to production of mucopolysaccharide acid hydrolyzed by testicular hyaluronidase by normal fibroblasts of rats' connective tissue. The concentration of mucopolysaccharides observed agrees with the data of authors who investigated these same types of tissues.

By the behavior of material previous hydrolized with hyaluronidase establishes the presence of mucopolysaccharide acid of hyaluronic and chondroitin sulphuric acid types. We can relate the turbidimetric data with the histochemical data with the presence of metachromatic granules.

The slightly higher values of neighboring tissue compared to those of distant connective tissue are in agreement with the results of authors who have found an increase of polysaccharides in the peritumoral tissue. Sarcoma fibroblasts do not produce "in vitro" mucopolysaccharides detectable by stains for meta-

chromasia although in the nutritive medium small quantities have been found by the turbidimetric method.

Our results obtained by cultivating pure sarcoma cells show that the mucopolysaccharides found by the other authors in total tumor extracts may have originated in the cells of the tumor stroma.

The fibroblastic tumor cell type differs from corresponding normal element by its low capacity to synthesize mucopolysaccharides. We believe that the small amount found in the culture medium of tumor fibroblasts could be due to a greater lability of the mucopolysaccharides synthesized by the tumor cells, or to a greater activity of their despolymerizing enzymes.

The observation realized with mixed cultures from normal and tumor tissues suggests that the sarcoma cell which, under these conditions, slows down its degenerating process would utilize the mucopolysaccharides synthesized by the normal cell for its growth.

The increase of mucopolysaccharides in the blood of cancerous patients cited by Mancini (13) and Deutsch (4) may be due to the destruction of stroma by the enzymes of the tumor and its liberation in the blood.

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BOOK REVIEWS

Perspectives in Virology. A Symposium. Ed., Morris Pollard. Published for the Institute of Microbiology, Rutgers University by John Wiley and Sons, Inc., New York, Chapman & Hall, Ltd., London. 1959. xix + 312 pp. Illus. Price

This book presents twenty papers given by leading European and American virologists and also discussions of these papers by forty-five virologists and oncologists during a symposium arranged in memory of the late Dr. F. R. Beaudette, formerly of Rutgers University. The contents of this monograph are most fittingly introduced by Dr. Selman A. Waksman who, in his foreword, has presented a moving picture of Dr. Beaudette as a great human being and an outstanding investigator.

The well prepared papers give a comprehensive summary of the present state of knowledge of the various aspects of plant, bacterial and mammalian viruses, including tumor viruses. They are well documented and each includes a representative list of pertinent literature references. The following aspects are discussed: chemical nature of plant viruses, genetic interaction of bacterial viruses and bacteria; a morphological, immunological and chemical study of animal virus multiplication; nutritional requirements of cells in virus propagation; quantitative analytical studies on host-virus relationship; implications of recent studies on measles virus and of the multitude of newly discovered human viruses to the development of vaccines and prevention of common viral diseases; natural history, epidemiology and specific immunity to viral infections. Each paper is followed by an excellent discussion which adds greatly to the discussed subjects.

A considerable part of the book is devoted to papers on the recent discoveries in the field of tumor viruses, followed by an extensive discussion, during which a number of participants have presented a considerable amount of new, as yet unpublished, observations. The reader will be impressed by the increased interest and participation of virologists in the study of neoplasia of animals and man. His interest will be further stimulated by the importance of new discoveries in virology and their application to the study of the relationship of viruses to the origin of cancer.

The book is an excellent proof of the extent of benefit which can be derived from a meeting in which authorities in the various aspects of the rapidly growing field of virology are able to participate and exchange recent information, in both their papers and in a well conducted discussion. It is therefore fortunate that the proceedings of the meeting, limited to approximately 120 participants, have now become available to the interested public. The book will be a valuable reference book for all virologists, research workers, teachers and students.

The program committee is to be congratulated on organizing an excellent and well-balanced symposium. The editor and publishers deserve special praise for this excellent publication. A review of this book would be far from

complete without a most grateful mention of Gustav Stern of Hartz Mountain Products Corporation, whose generous support made the Symposium and the publication of its proceedings possible.

L. Dmochowski

Ciba Foundation 10th Anniversary Symposium. Significant Trends in Medical Research. Ed. G. E. W. Wolstenholme, Cecelia O'Connor and Maeve O'Connor. 41 il. 356 pp. $9.50, Little, Brown and Co., Boston, Mass.

This fiftieth major volume published by J. & A. Churchill, Ltd., for the Ciba Foundation contains the transactions of a special symposium arranged in celebration of the tenth anniversary of the opening of the Ciba Foundation. It represents a consideration of those important and distinctive achievements in biologic research over the past ten years which the participants believed would prove to be most significant in the next ten years' advances in biologic research.

Twenty-nine distinguished contributors, seven of them Nobel Laureates, participated in the symposium. Each was given freedom to select his topic, to say exactly what he chose, to look back and to look ahead, and to indulge in speculation on many points. The result is a collection of "essays" (with discussions) that represent a most remarkable achievement on two scores. First, there is much valuable data presented in an authoritative and often brilliant manner—material presented sufficiently broadly that those not actually working in the area can become oriented as to the important things which are known and the important things which are unknown in the field. Second, the very selection of material contributes to a broad perspective in terms of appreciating the major advances in medical research that have taken place in the past ten years—and what we may look for in the next ten years.

This symposium represents a most valuable addition to our medical knowledge and can be enthusiastically recommended. Two groups of people, in particular, will profit from study of Significant Trends in Medical Research:

1. The biologist or medical scientist who wishes a rather detailed review of certain broad areas of biologic-medical research, and

2. Those scientists whose areas of major interest coincide or overlap with any of the major topics covered in the symposium—topics such as Molecular Structure in Relation to Biology and Medicine, Chemical Basis of Virus Multiplication, Genetics and Medicine, The Nature and Mechanism of Action of Hormones, Malignant Transformation: Its Mechanisms and Nature, and Factors Influencing the Substance and Dimension of Medical Research in the United States.

Howard C. Hopps, M.D.

Virus In the Cell. J. Gordon Cook, The Scientific Book Club, London, England, 205 pages, 27 illustrations, 12s 6d.

The predominant theme in this book concerns the viral diseases of man, and the influence of such diseases in lower animals, plants, insects, and

bacteria on the fate of man. Since this book is not intended as a reference text, some minor inaccuracies should be overlooked. Among the 27 illustrations are excellent electron micrographs of viruses which are authoritative examples of ultramicroscopy. The label "common cold virus" on one of them is difficult to accept. Interesting perspective reviews explain (1) the impact of Jenner in the war on smallpox, (2) the purpose and activities of the Common Cold Research Unit at Salisbury, England, and (3) events which culminated in the development of the "Salk vaccine" for poliomyelitis. A significant omission is the role of tumor viruses in animal disease, and the possible application of such information into the tumor problem in man. While the book does not show publication date, it records events up to 1955. The pace of viral investigations and their publication in the lay press is so rapid that some informed readers may consider this book to be "out of date." Scientists may be apt to be critical of a book in which technical information has been simplified; however, Dr. Cook interprets highlights in virology for the intelligent, inquisitive lay reader. It should serve to encourage increasing support and appreciation for this important field of inquiry from an informed public.

Morris Pollard

Virus Virulence and Pathogenicity. Ciba Foundation Study Group No. 4, in honor of Prof. J. Mulder. G. E. W. Wolstenholmes and C. M. O'Connor, eds., 114 pp., 13 il., $2.00. Little, Brown and Company, Boston, 1960.

The above symposium was held on June 15, 1959. Its central theme was the pathogenicity and virulence of influenza virus, and related problems among other viral agents. Six formal papers were presented around which the discussions of 17 promient virologists were recorded. The papers and the discussions were very well edited, so as to exclude impertinent information.

Following opening remarks by the Chairman, Sir Macfarlane Burnet, papers were presented by C. H. Stuart-Harris, the Definition and Measurement of Virus Virulence; R. H. Morgan, Host-cell Factors and Virus Virulence; C. H. Andrewes, The Effect on Virulence of Changes in Parasite and Host; J. Mulder, Broad Aspects of the Problem of Human Virulence in Influenza Viruses; E. D. Kilbourne, The Severity of Influenza as a Reciprocal of Host Susceptibility; and D. A. J. Tyrrell and F. E. Burkland, The Virulence for Man of Some Respiratory Viruses Passed in Tissue Cultures. The discussions centered around two topics (1) evidence for and against variation in virulence and human pathogenicity as occurs in nature, and (2) some rationale in the search for viral strains which might be of value as unaltered immunizing agents.

This book has an attractive list of references and is adequately indexed.

Morris Pollard

Essentials of Healthier Living. Justus J. Schifferes, John Wiley and Sons, New York, 1960, 335 pp., $5.50.

Intended as a college textbook in personal and community health this book can be recommended by physicians to questioning, intelligent patients whose

education did not include basic concepts in this field. The correct attitude toward the medical profession is illustrated by the following statement from the Foreword by Howard R. Craig, M.D., Director, the New York Academy of Medicine: "The health educator is primarily a teacher . . . but he is never a diagnostician or therapist. He is the colleague of the physician but not a substitute for him. The author of this book is a health educator."

A minimum of anatomical and physiological information forms the background for discussion of such subjects as physical fitness, exercise, fatigue, sleep, smoking, care of ears, eyes, skin and teeth. Thorough coverage of basic nutrition introduces a discussion of metabolism and weight. Reproduction, pregnancy, childbirth, love, marriage and family living are discussed frankly and objectively. Emotions, anxiety, mental health and illness, accidents, alcohol and narcotics are treated in a calm, matter-of-fact tone. The basic facts of community health, the organizations involved, insurance, health rackets and misleading advertising are well presented, but in too brief a form in the opinion of the reviewer.

The reader will acquire a substantial basis of facts for developing sound opinions but may be left with many unanswered questions. For example, antibiotics receive a more mention. The layman will depend on his doctor to tell him when and what antibiotics he should take, but may wonder why, especially why they are sometimes contraindicated. He may have heard talk regarding their limitations, their misuse, and the development of resistant stains of organisms, and would welcome a discussion of the general principles involved. The statement that the joints between the sacrum and the ilium are frequently disturbed, producing a troublesome "sacroiliac pain" provides another example of information which some readers would find insufficient. A young person who had not experienced this pain might not notice that in the anatomical diagram the sacrum and the pelvis are labelled but the ilium is not. An older reader might wish more information on the locations of the various joints which might cause this pain as well as why his doctor may have placed certain restrictions on his activity. These illustrations probably represent inconsequential points but may indicate something of the extent of coverage.

As a whole the author has produced a sound and reasonably well balanced treatise within the limits he set for himself.

J. Allen Scott. Sc.D.
Professor of Preventive Medicine

The Sense of Smell. Roy Bedichek. Doubleday, New York, $3.50, 1960.

Roy Bedichek who died in May 1959 was a most unusual man and his book is equally out of the ordinary. Frank Dobie in his foreword has written a good characterization of his life and qualifications. Without any scientific laboratory background but with a keen ability to sense objectively and to evaluate his observation he enjoyed nature as few persons can enjoy it. His prodigious memory for incidents occurring over many years together with his wide range of reading have combined to produce a fascinating study of the

place of olfaction in nature. Lacking anatomical knowledge he used his insatiable curiosity to gain by conversation and correspondence what he lacked. Within almost any paragraph therefore one may find personal observations, quotations from the Greeks, folklore, superstitions and scientific studies. All of these are evaluated critically and presented interestingly. Roy wrote very smoothly and he has produced both a good reference work and a piece of literature. Lacking an index the book must be read to be enjoyed.

John G. Sinclair

The Lifespan of Animals. Ciba Foundation Coloquia on Ageing. Vol. 5. Ed. G. E. W. Wolstenholme and M. O'Connor. Little, Brown and Company, Boston. $9.50, 324 pp. plus cumulative index to vol. 1–5.

Twenty-seven participants under the chairmanship of J. F. Danielli debate the criteria of the term lifespan, the correlated characteristics of ageing as drawn from actuarial tables, wild and domesticated animals, laboratory and zoo animals and commercial fisheries. Comparison with invertebrate life cycles is attempted. The attempt to show senescence as a result of some simple process, or of completely interdependent processes, indicates the inherent fallacies in studies of correlation tables and curves. The wide variety of life cycles as measured in death rates at given intervals shows that species require independent appraisal and that the factors operating in any one are multiple and to some degree quite independent. No attention is paid in this volume to the data on individuality of metabolic and biochemical pattern probably genetically determined in large part. Except for insurance purposes, therefore, statistical studies are of limited value.

Ciba Foundation is to be congratulated on their willingness to sponsor symposia in very debatable fields of study where sometimes the net progress is meager. It defines problems.

John G. Sinclair

CALLING ATTENTION TO

APRIL, 1960

Calling Attention To: Items of possible interest to scientists & physicians. *Antibiotics:* Anonymous summary of 7th annual symposium on antibiotics held December 1959 in Washington DC is offered (*Amer J Hosp Pharmacy* 17/77–97/60). LP Garrod introduces excellent discussion on antibiotics in medicine (*Brit Med Bull* 16/1–83/60), with 16 contributors. H Welch & F Marti-Ibanez relate *The Antibiotic Saga* (Medical Encyclopedia Co., New York, 1960, 150 pp., $3).

Program of Chicago April 10&15 meeting of Federation of American Societies for Experimental Biology includes 2654 reports, with abstracts now categorized with full author index (*Fed Proc* 19/1–421/60 for abstracts, & pp. 423–539 for program). You get your money's worth in the Federation! MO Lee & Staff edit well!
Just received is magnificent summary volume by C Heymans & E Neil on *Reflexogenic Areas of the Cardiovascular System* (J & A Churchill, London, 1958, 271 pp., 56s); why the delay in letting us know about it? It's a must on baro- and chemo-receptors, reviewing the great work since 1927 on the carotid sinus.

Clinical: G Asboe-Hansen discusses pathogenesis of lupus erythematosus & other mesenchymoses (*Acta Dermat-Ven* 39/337/59). L Brouha reviews effects of work & heat on heart & circulation (*Indust Med Surg* 29/114/60). GL Bryan & JW Rigney examine emotional behavior of airline passengers (*Aerospace Med* 31/123/60). AH Hunt reviews wound healing (*Proc Roy Soc Med* 53/41/60). CR Kleeman & Co review pyelonephritis (*Medicine* 39/3/60). M Masuda & Co find urinary aromatic metabolites in schizophrenia (*J Nerv Ment Dis* 130/125/60). JM McKenzie implies thyroid activator in hyperthyroidism (*J Clin Endocrin Metab* 20/380/60). AL Porterfield & EG Jaco edit good symposium on social knowledge in medicine (*J Health & Human Behavior* 1/1–55/60). PH Prose & E Sedles make histochemical study of atopic eczema (*J Invest Dermat* 34/149/60). W Siede & A Klamp recommend corticosteroids for virus hepatitis (*Deutsch Med Wchnschr* 85/333/Feb 26/60). Note Symposium on medical problems of adolescence (*Practitioner* 184/285–337/60). MA Wessel discusses pediatricians & adoption (*New Eng J Med* 262–446/Mch 3/60). VI Zavrazhnov suggests prophylactic & curative effects of glucose in myocarditis (*Pharmakol & Toxicol USSR* 23/24/60).

Preclinical: G Arden & YM Liu study responses of lateral geniculate body to light stimuli (*Acti Physiol Scand* 48/36, 49/60). CW Crane & A Neuberger report on digestion & absorption of protein in normal humans (*Biochem J* 74/313/60). D Dennis & NO Kaplan find both D & L lactic dehydrogenases (*J Biol Chem* 235/810/60—wow! 56 articles & 337 pages in single issue!). GH Glaser analyzes neurological status of newborn, on both neuromuscular & EEG activity (*Yale J Biol Med* 32/173/60). M Kitamura & Co report on

metabolism of p-aminobenzoic acid (*J Biochem* 47/60/60). AP Krueger & RF Smith go into biological mechanisms of air ion action (*J Gen Physiol* 43/533/60). B Kudryashov discusses physiological anticoagulation system (*Prob Med Chem USSR* 6/3/60). TE Lowe considers control of body fluid volume in humans (*Physics Med Biol* 4/126/59). NE Miller & H Barry well analyze motivational effects of drugs (*Psychopharmacol* 1/169/60). MF Moody & JD Robertson report fine structure of retinal photo-receptors (*J Biophys Biochem Cytol* 7/87/60). JB Nelson edits symposium on biology of pleuropneumonia-like organisms (*Ann NY Acad Sci* 79/305–758/60). EC Pollard edits symposium on inactivation of viruses (*Ann NY Acad Sci* 83/513-760/60). N Robinson reviews molecular size & shape in re insulin, serum albumins, lysozyme, collagen & hemoglobin (*J Pharm Pharmacol* 12/129/60). NR Sarker notes mechanism of clot lysis (*Nature* 185/624/Feb 27/60). TM Sonneborn reviews genes & cell differentiation (*Proc Nat Acad Sci* 46/149/60). WE Stone & Co show chemical concomitants of cerebral convulsive activity (*Neurol* 10/241/60). JL Strominger considers nucleotide acid anhydrides as intermediates in metabolic reactions (*Physiol Rev* 40/55/60). AB Tsipin & Co analyze central nervous system sensitivity to ionizing radiation (*Bull Exp Biol Med USSR* 49/83/60). A Wilson & RP Edkins discuss modification of duration of drug action (*J Pharm Pharmacol* 11 Suppl 44, 54, Dec 59).

General: Justice WO Douglas describes *America Challenged* on conformity & world leadership (Princeton Univ Press, 60, 74 pp, $2.5). EA Gall goes into medical eponyms (*Amer Sci* 48/51/60). G Kepes well edits keen symposium on *The Visual Arts Today* (*Daedalus* 89/1–268/60). AA Liebow & LL Waters eulogize Milton Charles Winternitz (1885–1959) & give his bibliography (*Yale J Biol Med* 32/143/59). JS Nicholas reminds us well of Nobelatant Ross Granville Harrison (1870–1959) who really developed tissue culture (*Science* 131/337/Feb 5/60).

MAY 1960

Calling Attention To: Items of possible interest to scientists and physicians.

Oral hypoglycemics: Two useful reviews: L. J. P. Duncan & J. D. Baird on compounds administered orally in the treatment of diabetes mellitus (*Pharmacol Rev* 12/91–158/60) & R. Levine & S. Berger on orally active hypoglycemic drugs (*Clin Pharmacol Therap* 1/227/60).

Preclinical: L. G. Augenstine edits *Bioenergetics: Considerations of Processes of Absorption, Stabilization, Transfer & Utilization* (Academic Press, New York 3, 1960, 450 pp, $10). J. B. E. Baker surveys effects of drugs on foeti (*Pharmacol Rev* 12/37–90/60). H. A. Bickerman & S. E. Itkin comment on evaluation of antitussives (*Clin Pharmacol Therap* 1/180/60). A. K. Done discusses antirheumatic action of salicylates (*ibid* p 141). M Florkin & H. S. Mason edit 1st 2 vols of great treatise on *Comparative Biochemistry*, Vol 1 on *Sources of Free Energy* & Vol 2 on *Free Energy & Biological Function* (Academic Press, New York 3, 1960, 600 pp, & 185 pp, $35). E Heftmann & E

Mosettig offer *Biochemistry of Steroids* (Reinhold, New York 22, 1960, 224 pp, $5.75). Sir Hans Krebs gives Walter Dixon Lecture on biochemical aspects of ketosis (*Proc Roy Soc Med* 73/71/60). L. Peters discusses renal tubular excretion of organic base (*Pharmacol Rev* 12/1–36/60). Of utmost importance is K. H. Pribram's review of theory in physiological psychology (*Ann Rev Psychol* 11/1/60). F. W. Putnam edits integrated account of *The Plasma Proteins*, Vol 1 on *Isolation, Characterization & Function* & Vol 2 on *Biosynthesis, Metabolism & Alterations in Disease*, including information on electrophoretic & chromatographic analysis, antibodies, glycoproteins, metal-binding proteins, lipoproteins, plasma enzymes, circulating hormones, blood coagulation, abnormal globulins & genetic alterations (Academic Press, New York 3, 1960, 2 vols, 420 & 480 pp, $26.50). K Rodahl, J. T. Nicholson & E. M. Brown edit comprehensive & practical *Bone as a Tissue* (McGraw-Hill, New York 36, 1960, 358 pp, $16). F. R. Stohlman edits *The Kinetics of Cellular Proliferation* (Grune & Stratton, New York 16, 1959, 472 pp, 167 illust., $5.75). S. E. Wright well reviews *The Metabolism of Cardiac Glycosides* (CC Thomas, Springfield, Ill., 1960, 94 pp, $4.75).

Clinical: K. L. Artiss considers *The Symptom as Communication in Schizophrenia* (Grune & Stratton, New York 16, 1959, 242 pp, $6). R Asher comments wisely on the importance of clinical sense (*Brit Med J* 1/985/Apr 2/60). D. Benazo describes Clinical use of hypothermia (*Anesth* 15/134/60). J. N. Berry & G. S. Sainani describe infantile cirrhosis (*J Indian Med Asso* 34/121/Feb 16/60). W. F. Bowers discusses *Interpersonal Relationships in the Hospital* (why not simply in hospitals? CC Thomas, Springfield, Ill., 1960, 136 pp, $5). A. J. Butt edits *Treatment of Urinary Lithiasis* (CC Thomas, Springfield, Ill., 1960, 600 pp, 222 illus, $21). J. W. Hurst edits concise *Cardiac Resuscitation* (CC Thomas, Springfield, Ill., 1960, 152 pp, $5.5). G. M. Knauf describes bioeffects of radar energy (*Aerosp Med* 31/225/60). H. J. Moersch & H. A. Anderson offer *Diagnosis & Treatment of Diseases of the Trachea & Bronchi* (CC Thomas, Springfield, Ill., 1960, 116 pp, $4.25). M. M. Ravitch discusses *Intussesception in Infants & Children* (CC Thomas, Springfield, Ill., 1960, 136 pp, 48 illus, $9.5). D. M. Spain edits comprehensive *Diagnosis & Treatment of Tumors of the Chest* (Grune & Stratton, New York 16, 1960, 384 pp, 162 illus, $14.75). H. B. Stoner & C. J. Threlfell consider *Biochemical Response to Injury* (CC Thomas, Springfield, Ill., 1960, 472 pp, 127 illus, $13). S.Takahara & Co describe hypocatalasemia, a newly recognized genetic carrièr status (*J Clin Invest* 39/610/60). J.Walker & A. C. Turnbull edit discussion on *Oxygen Supply to the Human Fetus* (CC Thomas, Springfield, Ill., 1960, 346 pp, 137 illus, $10.5). W. G. Walter discusses the subtle matter of control of consciousness (*Anesth* 15/105/60). I. S. Wright surveys progress & problems in coronary artery disease (*J Amer Geriat Soc* 8/237/60).

General: E. Callaway & G. C. Stone criticizes psycho-pharmacologic theories (*Clin Pharmacol Therap* 1/247/60). H. Clegg opens symposium on human experimentation *World Med J* 7/77/60). W. H. Hartung introduces interest-

ing historical review symposium on medicinal chemistry (*J Chem Educ* 37/168–201/60). I. & P. Opie describe *The Lore & Language of School Children* (Oxford Univ Press, New York, 1960, 417 pp, $8). W. T. Stearn revives memory of masters of biological illustration Franz (1758–1840) & Ferdinand (1760–1826) Bauer (*Endeavor* 19/27/60).

C. D. LEAKE

NOTES AND NEWS FROM THE MEDICAL BRANCH UNIVERSITY OF TEXAS, GALVESTON

Special lectures.

May 3, 4, 5. Kempner lectures. Wilder Pendield (Neurological Institute of Montreal).
The record of experience in the temporal lobe.
The importance of dreams.

May 9. Sigma Xi lecture. Harold G. Cassidy (Dept. of Chemistry Yale University).
The problem of the sciences and humanities: fission or fusion?

May 10. Kempner lecture and physiology seminar. R. F. Ogilvie (University of Edinburgh).
Human and experimental diabetes.
Medical education and study of pathology, University of Edinburgh.
The Nature of Otosclerosis.
Effect of growth hormone on endocrine organs in the rabbit.

May 12. McLaughlin lectures. David A. Rogers (Vanderbilt University).
Pulmonary complications of asian influenza.

May 28. Commencement Address.
President William V. Houston (The Rice Institute).

October 6, 7. McLaughlin lectures. Jack L. Strominger (Pharmacology Washington University).
Antibiotics as inhibitors of bacterial cell wall synthesis.
Mechanism of synthesis of sulphated mucopolysaccharides.

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